# Site Specific Immunosuppression for Promoting Vascularized Composite Allograft Survival and Reducing Systemic Immunosuppression Related Morbidity

by

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**Site Specific Immunosuppression for Promoting Vascularized Composite Allograft Survival and Reducing Systemic Immunosuppression Related Morbidity**

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Widespread clinical applicability of vascularized composite allotransplantation (VCA) has been limited by the high incidence of rejection and the requirement for systemic, lifelong, multi-drug maintenance immunosuppression for allograft survival that can lead to infectious and metabolic complications. Our goal was to evaluate a site-specific immunosuppressive strategy that promotes VCA graft survival and reduces the need for systemic immunosuppression.

Hand and face allografts provide the opportunity for site-specific delivery of immunosuppressive drugs given the accessibility, feasibility of visual monitoring. Conceivably, site-specific graft immunosuppression could provide therapeutic drug levels in the allograft, reduce systemic drug exposure and toxicity, and improve patient compliance and outcomes. This work attempts to develop effective drug delivery strategies for site-specific immunosuppression, while minimizing the need for systemic immunosuppression. We have described two strategies for site-specific immunosuppression in a rodent model of VCA. 1) Topical drug administration, and 2) locally implantable prolonged release formulation. These strategies were able to sustain the survival of VCA graft with minimal systemic immunosuppression.

We have developed a topical formulation of mycophenolic acid (MPA), and showed that MPA lipoderm (1%), and Tacrolimus (TAC) ointment (0.03%) provide high concentrations in the skin confirming the ability of targeting drugs to local tissues by topical administration, with low systemic concentrations. Combined treatment with topical TAC and MPA and low dose of

systemic TAC is effective in reducing systemic morbidity while sustaining VCA graft survival (>100 days). However, daily topical drug administration requires high patient compliance. We prepared a locally implantable formulation that consisted of TAC and polycaprolactone that can be placed in the allograft to provide sustained drug release over a prolonged period without any need for additional systemic immunosuppressive drugs. This approach prolonged VCA graft survival while minimizing the risk of systemic toxicity.

The results described in this dissertation show that replacement of systemic administration with site-specific immunosuppression is feasible and leads to better outcomes in a small animal model of VCA. Site-specific immunosuppression requires further investigation and development through pre-clinical and clinical research to achieve the optimal immunosuppression with minimal side effects. This will not only improve patient compliance and quality of life, but also increase the clinical applicability of VCA.

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## List of Abbreviations

| | |
|---|---|
| ATG | Anti-thymocyte globulin |
| AR | Acute rejection |
| AUC | Area under the blood concentration-time curve |
| R(ac) | Accumulation |
| AALAC | American Association for Accreditation of Laboratory Animal Care |
| ANOVA | Analysis of variance |
| BW | Body weight |
| CsA | Cyclosporine |
| CNI | Calcineurin inhibitors |
| C.V | Coefficient of variation |
| CrCl | Creatinine clearance |
| $C_{max}$ | Maximum blood level |
| $C_{trough}$ | Trough blood level |
| CL | Clearance |
| CR | Chronic rejection |
| DLN | Draining lymph nodes |
| DTx | Disk in the transplanted limb |
| DnonTx | Disk in non-transplanted limb |
| F | Bioavailability |
| FDCS | Franz diffusion cell system |
| GR | Glucocorticoid receptor |
| H&E | Hematoxylin and eosin |
| IACUC | Institutional Animal Care and Use Committee |
| IV | Intravenous |
| IL | Interleukin |
| IPGTT | Intraperitoneal glucose tolerance test |
| IFN-γ | Interferon gamma |

| | |
|---|---|
| IMPDH | Inosine monophosphate dehydrogenase |
| LLQ | Lower limit of quantification |
| LLE | Liquid-liquid extraction |
| LC/MS-MS | Liquid chromatography-tandem mass spectrometry |
| LTS | Long term surviving |
| MPA | Mycophenolic acid |
| MMF | Mycophenolic mofetil |
| MHC | Major histocompatibility complex |
| NF-AT | Nuclear factor of activated T cells |
| PCL | Polycaprolactone |
| PBS | Phosphate buffered saline solution |
| PRN | Pro re nata; b.i.d, bis in die |
| PCCA | Professional Compounding Centers of America |
| RAPA | Rapamycin |
| PBMCs | Peripheral blood mononuclear cell |
| RBC | Red blood cell |
| SOT | Solid organ transplantation |
| SD | Standard deviation |
| STAC | Systemic tacrolimus |
| SEM | Scanning electron microscopy |
| TAC | Tacrolimus |
| Tregs | Regulatory T cells |
| Tx | Transplanted |
| Non-Tx | Non-transplanted |
| UPMC | University of Pittsburgh Medical Center |
| Vd | Volume of distribution |
| VCA | Vascularized composite allotransplantation |

# 1.0 Introduction

## 1.1 Vascularized Composite Allotransplantation

Millions of Americans have sustained significant tissue loss due to trauma, disease, or congenital defects. Despite progress in surgical techniques, the severe injuries or defects requiring complex tissue reconstruction remain a clinical problem. Current treatment strategies involve autografting, flaps, and prostheses. These techniques are limited by significant complications including donor site morbidity, limited availability of autologous tissues, and complications of the intensive surgery [1-3]. Therefore, in most cases, the current reconstructive strategies are sub-optimal in terms of cosmetic and functional outcomes.

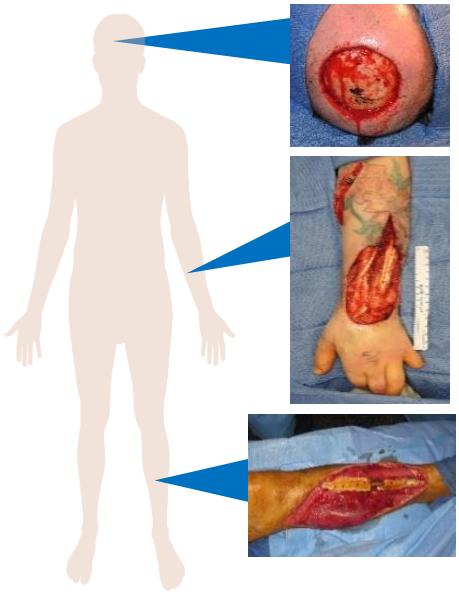


**Figure 1. Examples of common complex injuries requiring reconstruction.**

University of Pittsburgh School of Medicine, Department of Plastic Surgery. Used with permission

Vascularized composite allotransplantation (VCA) has the potential to replace like tissue with like for those patients with severe injuries or defects as shown in **Figure 1,** that cannot be reconstructed with conventional surgical procedures [4-6]. Vascularized composite allotransplantation is a procedure that is associated with immunologic and reconstructive challenges [7-10]. To date, more than 200 VCA procedures have been conducted worldwide with appropriate cosmetic and functional outcomes. This includes transplantation of extremities, face, abdominal wall, larynx/trachea, uterus, and penis [11, 12].

With more than 110 upper extremities transplants and 35 facial transplants performed worldwide, upper extremities and face transplantations are currently the most common types of VCA [13-16]. More than 185,000 amputations happen annually in the United States. Forty five percent of amputations are due to accidents. Seventy percent of traumatic amputations include the upper extremities [17-19]. Nearly1.7 million individuals are currently living with limb loss, and this number may increase by the year 2050 [17, 20]. Limb loss has significant psychosocial impact on patients in terms of aesthetic and functional aspects and has also economic impact in terms of the inability to do the daily life activities, and the high costs associated with the procedure [21-24]. Therapeutic option for patients with limb amputation or loss is prostheses. Many patients do not use their prostheses because they do not meet their needs. Only fifty percent of all upper extremity amputees receive prosthetic services. Thirty to fifty percent of the patients do not use the prostheses regularly [25].

Vascularized composite allotransplantation in the form of hand or arm transplantation provides a comprehensive reconstruction in terms of appearance and function and, thereby, can significantly enhance the quality of life of the recipient [12, 26] as shown in **Figure 2** [26]. The patient survival rate for hand transplantation is 98.5%, with an overall graft survival rate of 83.1%

at 5 years [14]. The high survival rates are attributed to advances in the field of transplantation, including organ preservation methods, surgical techniques, and development of effective immunosuppressive drug therapies [27]. Patient adherence to the immunosuppressive therapy is essential to reduce the risk of rejection and to improve the outcomes. Despite this success, VCA transplant recipients still face considerable challenges and complications that negatively impact the patient outcomes and limit wider clinical applications of the procedure. Successes and challenges in VCA are shown in **Table 1**. VCA is non-life saving procedure that is used to improve a patient's quality of life [28]; therefore, the goal is to investigate and develop novel strategies to maintain the allograft and minimize the potential risks associated with the procedure including the high incidence of acute rejection (AR) and the toxicity of systemic maintenance immunosuppression.

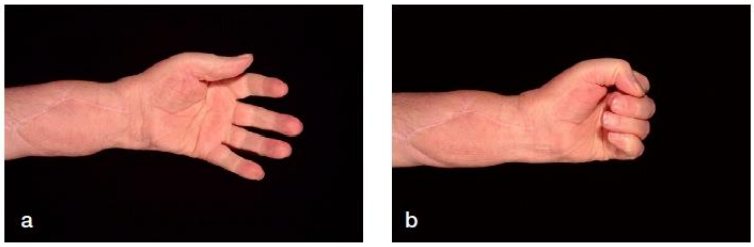



**Figure 2. A hand transplant patient at the five-year follow up. (a) extension (b) flexion. [With copyright permission from Kvernmo, H.D. et. al., 2005 [26]]**

**Table 1. Successes and challenges in VCA**

| Successes | Challenges |
|---|---|
| Promising cosmetic and functional outcome | Organ availability and preservation |
| Encouraging graft survival | Ischemia/reperfusion injury |
| Feasibility of visual monitoring | Acute skin rejection and chronic allograft deterioration |
| Feasibility of early detection of rejection | Long-term systemic maintenance immunosuppression |
| Accessibility for local drug application | Slow nerve regeneration |

## 1.2 Complications and Challenges in VCA

Vascularized composite allotransplant recipients are vulnerable to many complications and challenges that represent a hindrance for successful clinical outcomes and for wider clinical application of the procedure. High rates of AR rejection and the risks related to lifelong, systemic immunosuppression remain the greatest challenge and the most frequent complication of VCA.

### 1.2.1 VCA Susceptibility to Rejection

More than 80% of hand and face transplant recipients experience AR episodes in the first-year post-transplantation regardless of the use of maintenance immunosuppression [29-31]. The high incidence of AR episodes in VCA compared to solid organs, is probably due to the immunogenicity of VCA [32]. Vascularized composite allograft includes multiple tissue types with unique immunogenic properties such as skin, connective tissue, blood vessels, muscle, bone, and nerve tissue.

Skin has the highest immunogenicity and forms a major component in hand and face transplants, and thereby represents a major immunologic challenge [10, 32-34]. Many factors are responsible for the skin's susceptibility to rejection such as a high density of resident leukocytes and the immune-stimulating features related to the microvasculature [35]. Immune cell trafficking in the tissue affects skin's susceptibility to rejection. In vascularized skin allograft, immune cell trafficking depends on the lymphatic system which facilitates the lymphatic infiltration into the affected area, and thereby increases the susceptibility for rejection. Both the dendritic cells in the dermis and Langerhans cells in the epidermis present alloantigens and stimulate T-cells, leading to the initiation of alloimmune responses [36]. Once thought to be a subset of dendritic cells, recent

research suggests that Langerhans cells are a specialized subset of macrophages. Unlike dendritic cells which are short-lived and are derived from hematopoietic stem cells in the bone marrow, Langerhans cells are long lived, self-maintaining, and derived from embryonic precursors. Despite these differences, both types of cells play an important role in alloantigen recognition [37]. Skin composition also affects skin susceptibility to rejection. The dermis is characterized by high vascularization, a high density of lymphocyte adhesion molecules, and large number of dendritic cells [38]. Vascularized composite allograft also includes bone (vascularized bone marrow), which may affect the immune response (recipient) to the grafted tissue (donor) [39].

### 1.2.2 Acute and Chronic Rejection

Acute rejection (AR) is the most common type of rejection of VCA and can cause early allograft loss if it is not promptly treated. It is a T cell mediated immune response towards the donor MHC antigen resulting in inflammation, and lymphocytes infiltration into the donor allograft. It occurs within weeks to months after transplantation [40, 41], and can be detected by visual inspection of abnormal changes in the skin allograft and histopathological examination of skin biopsies [42]. Acute rejection is seen as a rash on the skin in hand and face transplantation and as inflammation in the mucosa of the mouth and nose in face transplantation [43]. Acute rejection has been reversible with the use of rescue systemic therapies including immunosuppressive drugs and corticosteroids and topical therapies in some cases [34].

Chronic rejection (CR) is an antibody or T cell mediated immune response towards the donor MHC antigen resulting in inflammation of the blood vessels, atherosclerosis, fibrosis, and allograft failure. It occurs within months to years after transplantation [44, 45]. There is always a possibility for CR due to several immune and non-immune risk factors that can cause allograft

injury [46]. Chronic rejection is irreversible and may require amputation. So far, CR has been reported in 9 patients with hand transplant and 2 patients with face transplant. Four of the hand transplants required removal of the allograft. Despite the low incidence of CR in hand and face transplants as compared to AR, close monitoring of the patients is necessary in order to maintain long-term allograft survival [47]. The observations on acute and chronic rejection in hand and face transplants confirm the need for novel immunosuppressive approaches to prevent rejection.

### 1.2.3 Mechanism and Consequences of Rejection in VCA

Although the mechanism of rejection in hand and face transplants is not completely identified, skin and vasculature are key targets for the immune system. Allograft rejection is local and cell-mediated, mainly driven by T lymphocytes [33, 48]. After transplantation, dendritic cells from the donor's skin migrate to the recipient's lymphatic nodes that drain the allograft. There, dendritic cells present the donor's antigens to the recipient T cells, which leads to the activation of T cells, and the initiation of rejection [49]. Rejection begins with a significant increase in lymphocytes infiltration around the blood vessels. The lymphocyte infiltrates are mainly composed of CD3+, CD68+, Foxp3+, and CD4+/CD8+ T cells [50, 51]. While the nature of the lymphocyte infiltrates remains the same, the expression of the lymphocyte adhesion markers in the endothelium and within the infiltrates significantly increase with the severity of rejection. The migration of the lymphocytes into the dermis and the trafficking in the epidermis lead to the destruction of the basal cells of the epidermis, and epithelial apoptosis and necrosis [52]. Cytokines (interleukin-1 α, interferon-γ, interleukin-2, interleukin-4, interleukin-5, and interleukin-10), chemokine, and adhesion molecules (LFA-1, ICAM-1, E-selectin, P-selectin, VE-cadherin, psoriasin, IDO, and Foxp-3) recruit more lymphocytes and other immune cells to initiate and

promote the immune response to the donor allograft [53-55]. IL-4 and IL-5 that are produced by Th2-type CD4 T cells promote the infiltration and proliferation of eosinophils at the allografted tissue. Eosinophils are involved in the formation of dermal fibrosis and vasculopathy, and the induction of CR [53]. Clinically, AR begins as skin lesions in the allograft. The progress of the skin rejection is due to the migration of immune cells from dermis to epidermis, and loss of the epidermal layer which is the final stage of skin rejection as shown in **Figure 3** [26].

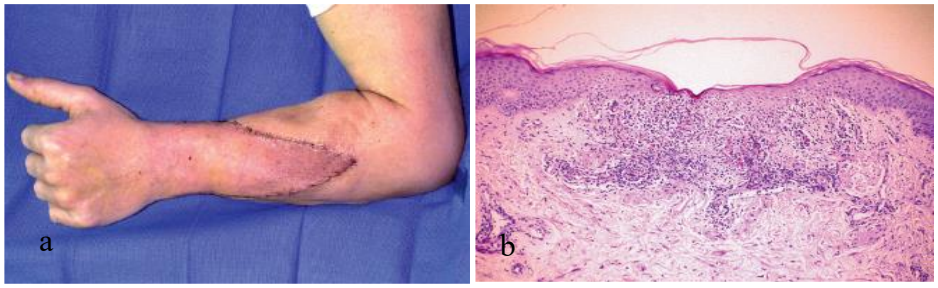



**Figure 3. Acute rejection in hand transplant patient : (a) Clinical signs of acute rejection characterized by rash and edema in the proximal triangular area. (b) Histological signs of acute rejection characterized by perivascular and dermal lymphocyte infiltration (H & E. x 40). [With copyright permission from Kvernmo, H.D. et. al., 2005 [26]]**

## Evaluation of Rejection in VCA

In contrast to the solid organs, hand and face transplants provide unique opportunity for early detection of rejection by visual observation of abnormal changes in the allograft [56]. Clinical findings can be correlated with histological findings. Acute rejection episodes are initially characterized by maculopapular rash followed by perivascular and dermal lymphocytic infiltration and mild epidermal degeneration [57]. Histological evaluation of rejection defined according to the established grading scale (Banff 2007) is described in **Table 2** [58, 59]. Histopathology or Banff 2007 working classification of rejection is still the gold standard for diagnosis of the acute rejection [60]. However, histopathology has some limitations including the latency between the tissue damage and the final diagnosis, and the limited ability to differentiate between skin rejection and skin inflammation as they involve similar mechanisms (molecular and cellular) and features [50, 51].

**Table 2. Histologic classification of skin-containing composite tissue allograft (Banff 2007)**

| Grade of skin rejection | Histological characteristics |
|---|---|
| Grade 0 | No lymphocytic infiltrate |
| Grade 1, mild rejection | Perivascular lymphocytic and eosinophilic infiltration |
| Grade 2, moderate rejection | Perivascular inflammation with or without mild epidermal and/or adnexal involvement |
| Grade 3, severe rejection | Dense inflammation and epidermal involvement with epithelial apoptosis |
| Grade 4, necrotizing rejection | Necrosis and loss of the epidermis |

### 1.2.5 Systemic Immunosuppressive Therapy

Acute rejection episodes could damage the allograft tissue and lead to allograft failure and loss of function. Immunosuppression is essential to prevent AR. VCA transplant recipients receive potent immunosuppressive drugs. The immunosuppressive drug regimens used in VCA are derived from solid organ transplant (SOT) protocols [61] and are shown in **Table 3** [62-64]. These drugs inhibit the immune response in a nonspecific way as shown in **Figure 4**. Hand and face transplant recipients receive induction therapy followed by maintenance therapy.

#### 1.2.5.1 Induction Therapy

Induction therapy consists of immunosuppressive agents that are administered pre-operatively, intra-operatively or post-operatively. The goal is to prevent AR episodes during the early post-transplantation period by providing intense immunosuppression at the time of transplantation [65, 66]. Induction therapy includes polyclonal antibodies (Anti-thymocyte Globulin, ATG) or monoclonal antibodies (basiliximab and alemtuzumab).

***Anti-thymocyte Globulin***

Anti-thymocyte Globulin (ATG) are non-human polyclonal antibodies produced by immunizing horses or rabbits with human lymphocytes. ATG causes depletion of circulating human T lymphocytes by multiple mechanisms including opsonization, phagocytosis, and lysis. The immunosuppressive effect of ATG is sustained for several months. ATG is non-human polyclonal antibodies, and thereby the possible side effects include cytokine release syndrome [65].

***Basiliximab***

Basiliximab is a humanized mouse monoclonal antibody to the α chain (CD25) of the IL-2 receptor on T cells. It is a competitive inhibitor of the IL-2 receptor. Inhibition of IL-2 receptor prevents T cell activation and proliferation while avoiding the side effects associated with opsonization, phagocytosis, and lysis of T cells. Basiliximab is a humanized mouse monoclonal antibody (70% human, 30% murine), and thereby it is less immunogenic than ATG. Studies have shown that basiliximab results in significantly lower rates of serum sickness, CMV infection, leukopenia, and thrombocytopenia as compared to rabbit ALG [67].

***Alemtuzumab***

Alemtuzumab (Campath) is a humanized mouse monoclonal antibody against human CD52 cell surface protein [68]. CD52 is expressed by B and T lymphocytes, monocytes, macrophages, and eosinophils. It increases antibody-dependent lysis and causes significant lymphocyte depletion. However, the mechanism of action is still under investigation. Alemtuzumab has been used for treating rheumatoid arthritis, vasculitis, multiple sclerosis, B-cell chronic lymphocytic leukaemia, and in bone marrow and kidney transplantation [69].

### 1.2.5.2 Maintenance Immunosuppression Therapy

Maintenance immunosuppressive therapy consists of multiple immunosuppressive agents that acts on different molecular targets. The goal of the combination therapy is to achieve efficient immunosuppression while reducing the side effects associated with individual drugs. The risk of AR is the highest in the first few months after transplantation. Therefore, high doses of immunosuppressive agents are prescribed during this period and doses are deceased with time post transplantation [70, 71]. Maintenance therapy normally includes a calcineurin inhibitor

(tacrolimus, TAC), an antiproliferative agent (mycophenolate mofetil, MMF), and anti-inflammatory agent (prednisolone) [64]. Doses of these agents are adjusted to prevent AR and to reduce systemic side effects. Corticosteroid boluses have been used as rescue therapy to treat >80% of AR episodes [29]. Increased systemic immunosuppression and topical tacrolimus and clobetasol have also been used as rescue therapy when steroids were ineffective [34, 52].

***Corticosteroid***

Corticosteroids are natural hormones that have immunosuppressive effect. Prednisone is a synthetic derivative of corticosteroids that has longer half-life, better stability, and reduced toxicity and thereby is the most commonly used steroid for suppressing immune response in transplantation. Corticosteroids modulate the immune system by binding intracellularly to the glucocorticoid receptor (GR). The glucocorticoid receptor becomes activated upon binding with the corticosteroid and enters the nucleus, where it can directly influence gene expression of pro-inflammatory and anti-inflammatory cytokines. Adverse effects of prednisone include increased risk of infections, impaired wound healing, osteoporosis, and cushing's syndrome [72, 73].

***Tacrolimus***

Tacrolimus (TAC) is a calcineurin inhibitor that is obtained from the streptomyces tsukubaensis bacterium, and it has become the main component of the immunosuppressive regimens in VCA [74]. TAC acts by inhibiting calcium-activated calcineurin, leading to decreased IL-2 mediated T cell activation. It enters the lymphocytes by passive diffusion and binds to FK binding protein-12. The formed complex binds to calcineurin which is a phosphatase responsible for the dephosphorylation of nuclear factor of activated T cells (NF-AT). This inhibits NF-AT translocation to the nucleus and thereby inhibits the production of IL-2, resulting in inhibition of T cell activation [75]. TAC has been shown to be more potent than cyclosporine (CsA), allowing

for TAC to be an alternative to CsA for preventing AR episodes, and allowing for TAC to be used as monotherapy in kidney transplantation [76, 77]. Adverse effects with TAC include infection, malignancy, diabetes, nephrotoxicity, and neurotoxicity [78, 79].

***Mycophenolic Acid***

Mycophenolic acid (MPA) is an antiproliferative agent. It is used due to its better specificity for T and B lymphocytes as compared to azathioprine [73, 80-82]. Mycophenolic acid is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), which is important enzyme in the de novo pathway for purine synthesis. Lymphocytes lack the alternate pathway for purine synthesis, the salvage pathway. This explains the safety, efficacy, and specificity of MPA on T and B lymphocytes [82]. Adverse effects include leukopenia, gastritis, esophagitis, and opportunistic CMV infection [73, 80].

**Table 3. Immunosuppressive drugs used in hand and face transplant recipients**

| Therapy type | Medication | Pharmacological class | Dosage and Administration |
|---|---|---|---|
| Induction therapy | Anti-thymocyte globulin (ATG) | T cells depleting agent (polyclonal antibodies) | Intravenous infusion, 1.5 mg/kg /day with the first dose given prior to transplantation |
| | Alemtuzumab (Campath) | T cells depleting agent (monoclonal antibodies) | Intravenous infusion, 1 × 30 mg |
| Maintenance therapy | Tacrolimus | Calcineurin inhibitor | Capsules 0.1-0.1trough blood concentrations of 5-15 ng/ml for first 1-5 months and 5–10 ng/mL thereafter |

| | | | |
|---|---|---|---|
| | Mycophenolate mofetil | Antiproliferative agent | Tablets (1-2 g/day), 1.7-4 µg/ml (+TAC), and 1.3-3 µg/ml (+CsA) |
| | Prednisolone | Steroids | Tablets (5–15 mg/day) |
| Rescue therapy | Glucocorticoid bolus<br>Increase in maintenance immunossupression<br>Topical tacrolimus (Protopic, 0.1, 0.03%) and clobetasol (Temovate) | | |

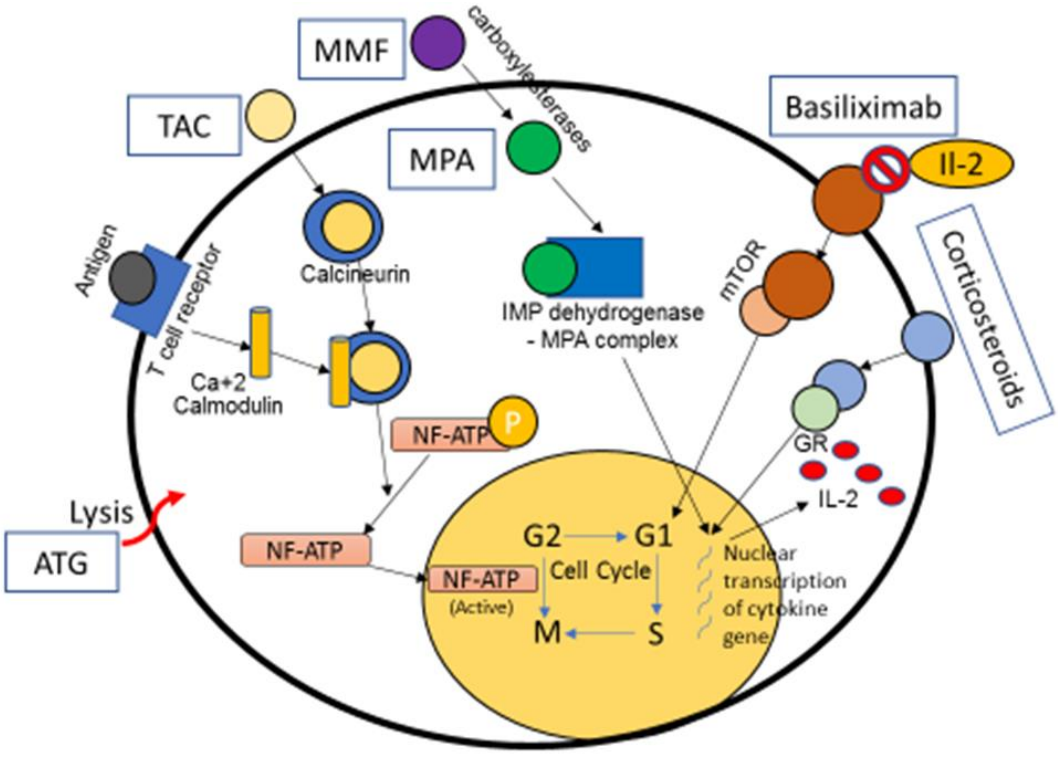



**Figure 4. Mechanisms of T cell-mediated immunosuppression by immunosuppressive drugs.**

#### 1.2.5.3 The Shortcomings of Current Immunosuppressive Treatments

Currently, systemic immunosuppressive agents are used to prevent rejection in VCA patients. Even though these are essential to maintain the allograft, the complications are increasingly being considered. These drugs cause chronic global immunosuppression which enhances the risk of opportunistic infections and malignancies [66, 83]. The side effects associated with systemic immunosuppressive drugs are also considerable in SOT patients. Some of the notable side effects are nephrotoxicity of calcineurin inhibitors (CNI), hypertension and cardiovascular adverse effects associated with corticosteroids [84-86], and gastrointestinal and genitourinary adverse effects associated with mycophenolate mofetil [87, 88]. Therefore, obviating the long-term use of systemic immunosuppressive drugs in transplant patients is increasingly being considered.

Skin is the most immunogenic component of VCA and mandates higher systemic maintenance immunosuppression than SOTs. This is associated with higher risks of systemic side effects. It has been reported that a higher incidents of infections and metabolic complications occur in VCA patients as compared to SOT patients because they undergo long-term immunosuppression at higher doses to prevent the rejection of highly antigenic tissues [81]. The other side effects are malignancies and nephrotoxicity. According to the international registry on hand and composite tissue transplantation (IRHCTT), 40% of the hand transplant recipients experience hyperglycemia, 26% experience nephrotoxicity, 32.3% experience bacterial infections, 18.5% experience viral infections, and 12.3% experience fungal infections [11]. The most common side effects associated with systemic use of immunosuppressive drugs in VCA patients are presented in **Table 4**. Moreover, systemic administration (intravenous or oral) of these drugs is usually associated with fluctuating blood levels of these drugs, leading to toxic or sub-therapeutic levels [89, 90]. Because

of this, each patient undergoing immunosuppressive treatment must be carefully monitored and have their dose adjusted based on blood concentrations.

**Table 4. Some of the common side effects associated with systemic administration of immunosuppressive drugs that contributed to the morbidity and mortality in VCA transplant recipients.**

| **Infection** | **Metabolic complications** | **Malignancy** |
|---|---|---|
| Ulnar osteomyelitis | Hyperglycemia | Lymphoproliferative disease |
| CMV infections | Diabetes | Squamous cell carcinoma |
| Cutaneous mycosis | Nephrotoxicity | Basal cell carcinoma |
| Cutaneous papilloma | Cushing's syndrome | |
| Herpes simplex | | |

Patient noncompliance and/or non-adherence to the prescribed medication regimens are other challenges. VCA patients are required to adhere to a complex dosage regimen of medications along with intensive physical therapy. With the long list of adverse effects and the different medications that a transplant patient must take, non-adherence rates are very high. Some studies estimate that up to 68% of the transplant patients do not adhere to their treatment regimen. However, there are several factors that can influence the level of non-adherence including socioeconomic status, sex, and education. Patient non-adherence to medication regimen is associated with poor clinical outcomes [91, 92]. The shortcomings described herein emphasize the significance of improving the current immunosuppressive therapies and developing safe and

effective strategies to improve patient and graft outcomes and reduces the need for daily systemic immunosuppression and its systemic complications. The next section of this chapter will describe one potential strategy specifically graft-specific delivery of immunosuppressive drugs.

### 1.3 Site-Specific Immunosuppression

Unlike solid organs, vascularized composite allografts are easily accessible for site-specific delivery of immunosuppressive drugs. Site-specific delivery of immunosuppressive drugs is one approach in VCA to increase the availability of current immunosuppressive drugs in the graft for site specific immunosuppression while decreasing the systemic availability and thereby reducing the systemic toxicity and increasing patient compliance [93, 94].

The idea of site-specific immunosuppression has been discussed in VCA. The first report of local drug delivery was demonstrated in a transplantation setting by Billingham et al. in 1951. He reported that topical cortisone administration prolonged rabbit skin allograft survival. After that, graft-specific drug delivery systems have been used in different animal models. Topical delivery of cyclosporine (CsA) to the skin allografts resulted in longer survival of Buffalo rat skin allograft in Lewis rats [95]. Previous study showed that continuous infusion of corticosteroid via renal artery significantly increased graft survival compared with systemic delivery in a rat renal transplant model [96]. Direct delivery of immunosuppressive drugs to the organ has been widely used in different animal models after successful results obtained from this study [96]. Local immunosuppression using tacrolimus administered via hepatic artery or portal vein of the transplanted liver significantly improved allograft salvage in Lewis-to-PVG rat liver transplantation model [97]. Later, survival benefit with limited systemic immunosuppression has been demonstrated in a canine model [98]. Local immunosuppression has been demonstrated successfully also in bowel [99] and in islet transplantation [100]. Additionally, other local systems, such as drug loaded polymer rods, matrices, and liposomes have been effectively used in animal models for site-specific immunosuppression. It has been also shown in preclinical and clinical lung transplantation that inhalation of TAC decreased cytokine production and NF-kB activation [101,

102]. Inhaled cyclosporine improved rejection–free survival in lung transplant recipients [103]. Despite the limited applicability of some of these drug delivery systems in VCA, these systems demonstrate the feasibility and efficacy of site-specific immunosuppression in transplantation in general.

Vascularized composite allografts are readily accessible for site-specific delivery of immunosuppressive drugs. They offer the possibility for topical, transdermal, or subcutaneous drug administration. Topical administration of immunosuppressive drugs is a potential therapeutic option with which to provide site specific immunosuppression, with minimal risk of systemic exposure and toxicity [104]. Advantages of topical drug administration versus systemic drug administration are shown in **Table 5**. In an earlier work, our group and others reported that topical TAC prolonged allograft (hind limb and face) survival after a short course of systemic therapy with CsA resulting in low systemic levels of CsA in rat model of VCA [104, 105]. However, these topical agents while Food and Drug Administration (FDA) approved, are used off-label in treating AR episodes as adjunctive to the systemic therapy in clinical VCA [30].A systemic evaluation of transdermal drug administration in VCA has not been reported yet. Subcutaneous drug administration has been reported in VCA and is mainly depend on the use of polymer-based drug delivery systems for controlled drug release. Biomaterials-based controlled release systems hold significant potential in VCA. These systems could provide controlled delivery of drugs directly to the allograft over a prolonged period and effectively inhibits the rejection, while mitigating the complications of systemic immunosuppression. These drug delivery systems should be made from biocompatible and biodegradable materials that should not produce toxic acidic derivatives during biodegradation that could result in local inflammation or foreign body reactions. They should be able to encapsulate and deliver a wide range of drugs, and should be easily produced,

administrated, and removed when necessary. Our group also reported the use of locally implanted polymeric disks at the allografts to achieve long-term graft survival in a rat hind limb allotransplantation model. However, the initial burst drug release followed by high blood levels throughout the study [106] made it difficult to confirm whether local or systemic immunosuppression was responsible for preventing the allograft rejection. This necessitated re-designing of the disk to control drug release and provide site specific immunosuppression.

Studies have referred to the importance of the donor graft site in forming the immune responses [107, 108]. Many of the priming immune mechanisms that lead to the formation of the alloimmune response occur in the donor graft site [108]. Delivery of drugs directly into the allograft may modulate the recruitment of immune cells and their interaction with donor antigen presenting cells and may have stronger effects on the early alloimmune responses with reduced systemic exposure to inhibit the systemic immune responses. All these results were obtained in preclinical experiments and confirmed that site-specific immunosuppression is a feasible and effective alternative approach to control allograft rejection and reduce systemic toxicity. More studies are needed to obtain robust preclinical data on the therapeutic strategies of application, pharmacokinetics and tissue distribution of the drugs, toxicity, and in-vivo local immunomodulation. The above information will help to develop novel drug delivery strategies and formulations for therapeutic delivery in VCA, and to design future clinical trials in VCA patients.

**Table 5. Advantages of using topical drug administration over systemic administration in VCA**

| Topical drug delivery | Systemic drug delivery |
|---|---|
| Good alternative for oral route for VCA application. | Unsuitable route of administration in certain situations (e.g. vomiting and diarrhea) |
| Avoidance of first pass metabolism | Oral route increases risk of first pass intestinal/liver metabolism, while parenteral route eliminates it |
| Local and sustained drug release over a prolonged period of time | Drug is systemically bioavailable for limited period of time |
| Decreased dosing amount and frequency | Increased dosing amount and frequency to achieve the therapeutic concentration at the site of action |
| Reduced systemic exposure and lower risk of systemic side effects | Increased systemic exposure and risk of systemic side effects |
| Lower risk of drug interactions | Increased risk of drug interactions |
| Increased patient compliance to the therapy | Relatively low patient compliance to the therapy |

## 1.4 Summary and Introduction to Dissertation

Vascularized composite allotransplantation has become a useful treatment option for severe tissue injuries or defects due to trauma, disease, or congenital defects. The skin component of VCA is highly antigenic and mandates daily intake of systemic immunosuppressive drugs to prevent rejection. The high incidence of rejection and the requirement for systemic, lifelong, multi-drug maintenance immunosuppression are the main challenges preventing a wider clinical application of VCA. Site-specific immunosuppression using safe and effective drug delivery strategies may help to overcome these challenges, increase therapeutic efficacy while reducing complications of systemic immunosuppression, considering that these transplants provide a unique opportunity for the use of site-specific immunosuppression and allow for easy monitoring. The central hypothesis guiding this project is that site specific graft immunosuppression provides high loco-regional concentrations of immunosuppressive drugs in the graft to inhibit the local immune response and sustain allograft survival. This will also lead to a reduction in systemic immunosuppressive drug levels, and thereby minimize the risk of systemic side effects and improve patient compliance.

Topical administration of immunosuppressive drugs is a potential therapeutic choice with which to provide site- specific immunosuppression, with minimal risk of systemic exposure and toxicity. Topical TAC (Protopic®) has successfully been used in clinical VCA to resolve AR episodes. There are, however, no data on the pharmacokinetics and tissue distribution of topically applied TAC in the VCA setting. We hypothesized that topical delivery of TAC (Protopic® 0.03%) will provide high concentrations at the site of application for local effect while decreasing systemic exposure and off-target effects. **Chapter 2** details the feasibility and superiority of topical TAC in comparison to systemic TAC administration in rats. The goal of the study was to evaluate the

ability of topical TAC (Protopic® 0.03%) at a dose of 0.5mg/kg/day to achieve high tissue concentrations at the site of application for local effects with low systemic concentrations. We assessed the pharmacokinetics and tissue distribution of topical tacrolimus (Protopic®, 0.03%) after single or repeated once-daily topical application in comparison to those after systemic delivery.

We successfully developed for the first time, a topical formulation of mycophenolic acid (MPA), a widely used immunosuppressive drug in SOT and VCA immunosuppression protocols. We hypothesized that topical delivery of MPA will provide high concentrations at the site of application for local effect while decreasing systemic exposure and consequentially off-target effects. **Chapter 3** details the feasibility and superiority of topical MPA in comparison to those after systemic administration in rats. The goal of the study was to prepare a topical formulation of MPA with good in vitro / in vivo characteristics such as release, permeation, and tissue bioavailability for further safety and efficacy evaluation in clinical VCA.

We hypothesized that combined treatment of topical TAC and MPA, in conjunction with low dose of systemic immunosuppression with TAC will sustain the allograft survival while minimizing risk of systemic side effects. Combining topical and systemic immunotherapy will provide therapeutic levels to effectively inhibit both local (donor allograft) and systemic (recipient) immune response mechanisms. Combining topical TAC and MPA will provide powerful local immunosuppression by effectively targeting multiple local pathways and targets and thereby selectively protect the skin from acute rejection. **Chapter 4** details safety and efficacy study of topical delivery of TAC and MPA, in conjunction with low dose systemic TAC in a rat VCA model. The goal of the study was to evaluate whether combined treatment of topical TAC and MPA applied on the allograft in conjunction with low dose systemic immunosuppression with

TAC can be effective in sustaining VCA graft survival and in reducing systemic morbidity in a low-dose combination corticosteroid-free regimen in a clinically relevant model of VCA.

A drawback for practical applications of topical agents is the need for daily application to provide efficacy which can lead to low adherence. Biomaterials-based controlled release system can control drug release and deliver the drug to the target sites over a prolonged period of time and thus increasing the therapeutic benefit and patient compliance while minimizing systemic toxicity. **Chapter 5** details feasibility and efficacy study of controlled delivery of tacrolimus directly to the allograft with a single TAC disk over prolonged period effectively inhibits immune rejection and prolongs VCA allograft survival via site-specific immunosuppression, while mitigating the complications of systemic immunosuppression. The goal of the study was to evaluate a site-specific immunosuppressive strategy that can promote VCA allograft survival and reduce the need for daily systemic immunosuppression using a novel technology of drug-eluting biomaterials in the allograft. Summary of major findings, conclusions, limitations, and the recommended future directions are discussed in **Chapter 6**.

## 2.0 Tacrolimus for Topical Immunosuppression in Vascularized Composite Allotransplantation: Evaluation of Pharmacokinetics and Tissue Distribution

## 2.1 Abstract

Skin is the most antigenic tissue of VCA and the primary target of rejection. High doses of oral TAC are administered to prevent AR, but this is associated with systemic adverse effects. Topical tacrolimus (Protopic®) has been used successfully in VCA, off-label, to treat AR episodes pro re nata (PRN). Systemic exposure to TAC following topical application is low and unlikely to result in systemic immunosuppression. There are, however, no data on the pharmacokinetics and tissue distribution of TAC after topical administration in the VCA setting. In this study, we evaluated the ability of topical TAC (Protopic® 0.03%) at a dose of 0.5mg/kg/day to achieve high tissue concentrations at the site of application for local effects with low systemic concentrations. We assessed the pharmacokinetics and tissue distribution of topical tacrolimus (Protopic®, 0.03%) after single or repeated topical application in comparison to those after systemic delivery in rats. Animals received either a single topical application of TAC ointment (Protopic®, 0.03%) (Group 1) or an intravenous (IV) injection of TAC (Group 2) at a dose of 0.5 mg/kg. In another experiment, animals received either daily topical application of TAC ointment (Protopic®, 0.03%) (Group 3), or daily intraperitoneal (IP) injection of TAC (Group 4) at a dose of 0.5 mg/kg for 7 days. Tacrolimus concentrations in blood and tissues were analyzed by Liquid Chromatography–Mass Spectrometry (LC/MS-MS). Systemic exposure to TAC following topical application was low and unlikely to result in systemic immunosuppression. Topical bioavailability of TAC was 11% of those achieved after systemic administration. There was a moderate drug accumulation with repeated once daily application, however all trough blood levels were sub-therapeutic (<3ng/ml). Topical application of TAC ointment (Protopic®, 0.03%) at a dose of 0.5 mg/kg/day provided high concentrations in the skin and confirmed the feasibility of targeting certain tissues by topical delivery with low systemic exposure. Tacrolimus ointment (Protopic®, 0.03%) is expected to be

a well-tolerated formulation for local delivery of TAC. This study confirms the feasibility of topical application of TAC for site specific graft immunosuppression and enables future applications in VCA.

## 2.2 Introduction

Tacrolimus has been widely used as a calcineurin inhibitor in the prevention and treatment of rejection in SOT patients [109, 110]. It has been used in triple drug therapy regimens in combination with MMF and prednisone in SOT [109-111] and in VCA [56, 112, 113]. TAC (Prograf®, Astellas Pharma Inc) is available as oral capsules and as an IV solution. The oral bioavailability of TAC is 25-30% in transplant patients due primarily to intestinal and hepatic metabolism [114, 115]. Oral doses of TAC are prescribed for VCA patients to achieve therapeutic levels at the target sites of rejection i.e., lymphocytes [116], and allograft, particularly skin [117]. Use of oral TAC is associated with serious metabolic side effects, infections, and malignancy [30, 118-121]. VCA offer unique opportunities for graft access for any treatment (site-specific therapies) and graft monitoring [104, 122]. Topical administration of TAC, via the skin or mucosa in VCA could improve effectiveness of the drug by predominantly concentrating the drug in the graft particularly in the skin, decrease systemic exposure, and consequentially off-target effects. Topical formulation for TAC is available (Protopic™) in two concentrations 0.1%, 0.03% (Astellas Pharma Inc), and has been proven as a safe and effective therapy for immune mediated dermatological conditions [123-125] such as atopic dermatitis, contact allergic dermatitis, and psoriasis [124-129]. Systemic exposure (AUC) of TAC from Protopic™ 0.03%, 0.1% ointment in atopic dermatitis patients approximately 0.5% of the values observed with oral doses in kidney and liver transplant patients [130, 131]. This makes topical TAC an attractive choice for treating AR in skin-containing VCA transplants as they share the same targets of therapy. After topical application, TAC locally acts on epidermal dendritic cells, a key cell in the initiation of rejection of skin. It inhibits expression of MHC-II antigen and prevents maturation of dendritic cells [132,

133]. It also inhibits expression of co-stimulatory molecules [134] and causes depletion of T cells [135].

Use of topical immunosuppressants has been reported in experimental models of VCA. Tacrolimus ointment applied every two days prolonged skin allograft survival [136]. Tacrolimus levels were variable. Our group reported that tacrolimus ointment (Protopic®, 0.1%) prolonged allograft survival after a short course of systemic therapy with CsA [104]. It has also been used in clinical VCA, off-label, to treat AR episodes pro re nata (PRN) [11]. Tacrolimus ointment b.i.d (Protopic®, 0.1%) and clobetasol cream b.i.d (Temovate ®, 0.05%) were effective in reversing AR in 5 hand transplant recipients [137], in 5 transplant recipients (4 face and 1 hand transplant recipient) in 6 hand transplant recipients [138], and 1 face transplant recipient [139]. It has been reported that grade 1-2 rejection could be treated with topical tacrolimus and clobetasol without increasing systemic immunosuppression levels. The skin biopsies revealed significantly decreased lymphocyte infiltration. These studies show that topical drugs can provide local effects. However, conclusions from these studies are limited by confounding elevated systemic levels. The pharmacokinetics and tissue distribution of TAC (Protopic®) after single and repeated topical application have not been evaluated in VCA. Determining the drug concentrations in the target tissues and its relation to blood levels may provide an idea about the therapeutic effects in VCA and the possible risk for side effects.

We hypothesized that topical delivery of TAC (Protopic® 0.03%) will provide high concentrations at the site of application for local effect while decreasing systemic exposure and off-target effects. The objective of this study was to assess the pharmacokinetics and tissue distribution of topical tacrolimus (Protopic®, 0.03%) at a dose of 0.5mg/kg/day after single or repeated topical application in comparison to those after systemic delivery in rats.

## 2.3 Materials and Methods

### 2.3.1 Animals

Experiments were performed in accordance with a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Animals (Male Lewis (Lew) rats aged 8 to 10 weeks and weighting from 300 to 320g at the time of dosing) were housed in a specific pathogen-free barrier facility and maintained in accordance with IACUC guidelines. All procedures were in a compliance with American Association for the Accreditation of Laboratory Animal Care (AALAC) recommendations and the principles set forth in the National Institute of Health Publication 80-23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended. Animals were housed individually, and plastic Elizabethan collars were used to prevent oral ingestion of the topical formulations and to prevent animal access to the application site.

### 2.3.2 Chemicals and Reagents

The chemical structures of tacrolimus and the internal standard, ascomycin (FK520), are presented in **Figure 5**. Tacrolimus was prepared in vehicle of 0.8% ethanol, 0.2% Cremophor EL (kolliphore®), and saline solution (Sodium chloride, 0.9% w/v, USP) for IV or IP administration in a final concentration of 1 mg of tacrolimus/ml. Tacrolimus solution was administered at a volume of 0.5 ml/kg for a dose of 0.5mg/kg. Tacrolimus ointment (Protopic®, 0.03%) was applied at 1.6g/kg for a dose of 0.5mg/kg. Tacrolimus powder, Cremophor EL (kolliphore®), and ethanol was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tacrolimus ointment (Protopic®,

0.03%) was commercially prepared by Astellas Pharma US in a base of mineral oil, paraffin, propylene carbonate, white 35 petrolatum, and white wax. The ointment was applied evenly on the hind limb of rat with massaging.

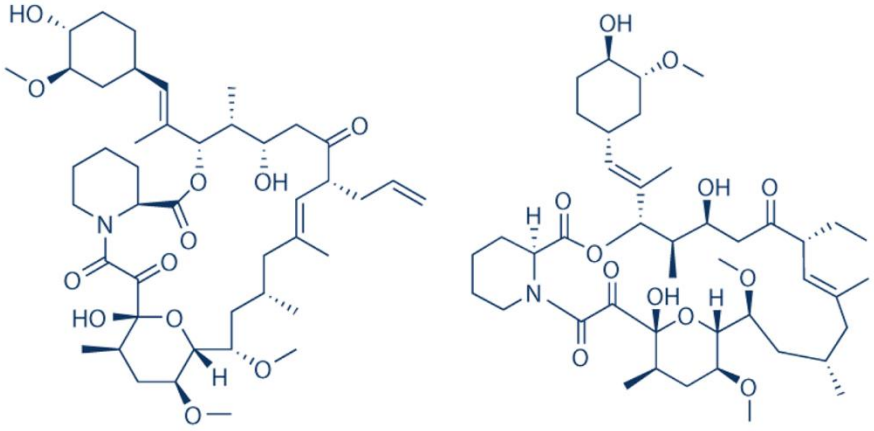


**Figure 5. Chemical structures of tacrolimus (right, molecular weight: 804.018 g/mol) and the internal standard, ascomycin (left, molecular weight: 792.0 g/mol).**

### 2.3.3 Pharmacokinetics and tissue distribution after single topical application of TAC ointment

The experiment was performed to evaluate drug exposure in local tissues and blood after single topical application of TAC ointment. Lewis rats were assigned to two groups. Animals received either a single topical application of TAC ointment (Protopic®, 0.03%) (Group 1), or an IV injection (Group 2) administered at an effective dose of 0.5 mg/kg. TAC ointment was applied on the right or left hind limb of the rat. Blood samples were collected by tail vein bleeding at 0, 2, 4, 6, 8, 12, and 24 hours after drug administration and were analyzed by LC-MS/MS for TAC concentration. Twenty-four hours after administrating the last dose, animals were sacrificed and

skin, muscle, and draining lymph nodes (DLNs) were collected from the application limb and the contralateral limb for measurement of TAC concentration.

### 2.3.4 Pharmacokinetics and tissue distribution after repeated topical application of TAC ointment

In another experiment, the drug exposure (drug accumulation) in local tissues and blood after repeated application (once daily for 7 days) was evaluated. Animals were allocated to two groups. Animals received either topical doses of TAC ointment (Protopic®, 0.03%) applied daily at an effective dose of 0.5 mg/kg, (Group 3), or IP injection in a dose of 0.5 mg/kg/day (Group 4). Blood samples were collected daily before administrating the next dose for trough concentration measurement. Twenty-four hours after administrating the last dose, animals were sacrificed and tissues (skin, muscle, and DLNs) were collected from the application limb and the contralateral limb for measurement of TAC concentrations. Skin at the application site was examined for any signs of irritation.

### 2.3.5 Local drug distribution in the skin and muscle after single topical application of TAC ointment

In another experiment, the local drug distribution in skin and muscle over 24 hours after topical application of TAC ointment was evaluated. Animals (n=4) received a single topical dose of TAC ointment (Protopic®, 0.03%) applied at an effective dose of 0.5 mg/kg. Biopsies from skin and muscle were collected at 2, 6, 12, and 24 hours post dose administration for measurement of

tissue concentration. Before excising tissue samples, the skin was wiped with ethanol-soaked gauze. Blood and tissue concentrations of TAC were analyzed by LC-MS/MS method.

### 2.3.6 Quantification of TAC in blood by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Fifty microliters of blood containing an unknown concentration of tacrolimus was added to a conical centrifugation tube, followed by two hundred microliters of a solution of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) to precipitate blood proteins. Five hundred microliter of acetonitrile containing an internal standard (ascomycin) at a concentration of 15ng/ml was then added and the mixture was vortexed at 3000 rpm for 2 minutes. Samples were centrifuged at 13,000 rpm for 3 minutes with the supernatant being poured off and collected into LCMS vials for analysis. Analysis was performed using a validated, reverse phased method for the detection of TAC in blood on a Waters micromass Quattro micro API mass spectrometer operated in a positive electrospray ionization mode, utilizing multiple reaction monitoring, after injection of 20 µL of sample. The Waters 2795 Alliance Separations Module was equipped with a nova-pack® C18 column, 2.1 x 10 mm cartridge (Waters # 186003523) heated to 55° C. Analytes were effectively separated using a gradient elution consisting of an aqueous mobile phase (95% $H_2O$ / 5% MeOH) and an organic mobile phase (100% MeOH), at a flow rate of 0.6 mL per minute. Mobile phases also contained 0.1% formic acid ($CH_2O_2$) and 2mM ammonium acetate. Monitored parent to product mass transitions for TAC and ascomycin were 821.63 → 768.33 and 809 → 756 m/z, respectively. TAC had a retention time of 1.2 minutes. The standard curve was linear for concentrations ranging from the limit of quantification (LoQ) value of 2 ng/ml up to concentration values as high as 40 ng/mL with an $R^2$ value of 0.9996 (With the lower limit for $R^2$ acceptability being defined as 0.99). Limit

of detection (LoD) was 0.1 ng/ml. Both intra- and inter-day precision were acceptable (C.V. <10%, n=3) at concentrations of 4.3, 15.7, and 24.6 ng/mL [140].

### 2.3.7 Quantification of TAC Concentration in Tissues

The skin sites for tissue sampling were wiped down three times with ethanol-soaked gauze to remove residual ointment on the surface. Skin and muscle were frozen with liquid nitrogen and pulverized in pestle and mortor to fragment the frozen tissues samples into fine pieces. Tissues were weighted and homogenized with cold methanol (1ml) in homogenization tubes using Mini-BeadBeater-1 (Cole-Parmer North America) for cell disruption. The homogenate was sonicated for 1 hour at 25 °C and then kept overnight at 4°C to allow for the complete extraction of the drug from the tissues. The homogenate was transferred to an appropriately labeled micro centrifuge tube and centrifuged at 2100 ± 100 rpm for 10 min. The supernatant was transferred to a labeled glass vial and evaporated by sample concentrator and the drug residue was reconstituted with blood (1ml). Tissue drug concentrations are expressed as ng/g of tissue weight. Extraction recovery of TAC from skin and muscle were 87% and 89%. To control for residual ointment on the skin, ointment was applied on limbs (n=4) and immediately cleaned with ethanol-soaked gauze. Biopsies from skin were collected and analyzed for TAC concentration. The highest TAC concentrations from residual ointment that remained on the skin after wiping off were minimal (19±9ng/g) compared to the actual tissue concentrations.

### 2.3.8 Pharmacokinetic Analysis

Descriptive pharmacokinetic parameters for TAC after topical and/or systemic administration were estimated by non-compartmental analysis using Phoenix WinNonlin® 6.1 (Certara, St. Louis, MO). The following pharmacokinetic/exposure parameters were obtained directly from the concentration-time profiles: Observed maximum blood level ($C_{max}$), and observed trough blood level ($C_{trough}$), area under the blood concentration-time curve extrapolated to time infinity, $AUC_{0-\infty}$ (calculated as $AUC_{0-t} + C_{et/\lambda z}$, where Ct is concentration at time t and $\lambda z$ is the slope of the terminal elimination phase). The bioavailability of TAC after topical administration was obtained as F = ($AUC_{0-\infty}$ topical). (i.v. dose)/($AUC_{0-\infty}$ i.v.).(Topical dose). Tissue to blood concentration ratios were obtained as tissue concentration/blood concentration. The accumulation ratio R(ac) was obtained as $AUC_{0-t,\ ss}$ after repeated once-daily doses/ $AUC_{0-t,\ 1}$ after first dose. The cut off values for non-, weak, moderate, and strong accumulation can be set at $R(ac) < 1.2$, $1.2 \leq R(ac) < 2$, $2 \leq R(ac) < 5$, and $R(ac) \geq 5$, respectively [141].

### 2.3.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 statistical software for windows (GraphPad Software, La Jolla, CA, USA). Data sets were checked for normality. Student t test, Mann Whitney test, or Wilcoxon Matched pairs test was used for two groups, and analysis of variance (ANOVA) was used when one independent variable with greater than two conditions or treatments and outcomes was evaluated and compared. Post hoc test (Tukey) was used to do multiple comparisons. All experimental results were expressed as the mean ± standard deviation (SD). A p value $< 0.05$ was considered as statistically significant difference. Statistically

significant data were presented as follows: *P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. Statistical tests are specifically indicated under each figure.

## 2.4 Results

### 2.4.1 Single topical application of tacrolimus (0.03% ointment, 0.5mg/kg) results in significantly lower systemic exposure, as compared to systemic administration of the same dose

In-vivo pharmacokinetic studies were performed to evaluate the potential for systemic exposure to TAC when the product was administered topically. Furthermore, these studies evaluated the tissue TAC concentrations achieved following product exposure, which can impact the product efficacy. The mean blood concentration-time profile of TAC after single topical application (0.03 % ointment, 0.5mg/kg) or IV bolus injection (0.5mg/kg) is shown in **Figure 6**. Following a IV bolus dose, TAC concentrations were high initially (40.6 ± 4.3 ng/ml), with concentrations declining quickly thereafter over time to reach low values (2.5 ± 0.4 ng/ml) at 24 hours. Following a topical dose, peak TAC concentrations (2±0.5 ng/ml) were reached between 2 and 4 hours. Concentrations were low, and the lowest values (0.3 ± 0.1 ng/ml) were achieved at 24 hours. The comparative non-compartmental pharmacokinetic exposure parameters of TAC derived from the blood concentrations-time data after topical and/or systemic administration including $AUC_{0-\infty}$, $C_{max}$, $C_{trough}$, and F after single topical application of TAC are presented in **Table 6**. The $AUC_{0-\infty}$, $C_{max}$, and $C_{trough}$ of TAC after topical administration were markedly lower than the values obtained after IV bolus of the same dose ($p<0.0001$). Bioavailability of TAC after topical administration was 11%.

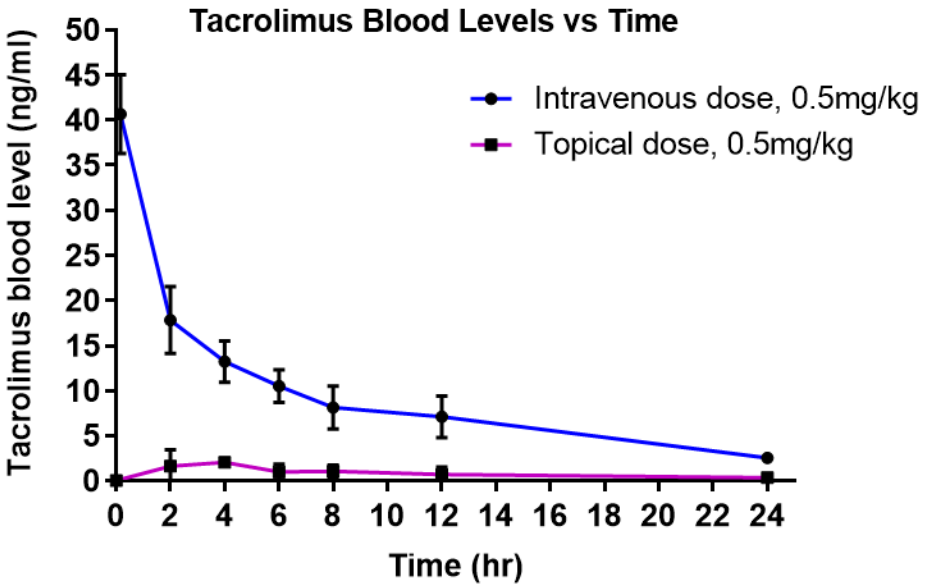



**Figure 6. Tacrolimus blood concentration–time profiles following single topical application of tacrolimus (0.03 % ointment, 0.5mg/kg) or IV bolus (0.5mg/kg). Data shown as mean+SD, n=6/Systemic TAC group, and 5/Topical TAC group.**

**Table 6. Comparative non-compartmental pharmacokinetic exposure parameters (mean + s.d.) following single application of TAC (0.03 % ointment, 0.5mg/kg) or IV bolus of TAC (0.5mg/kg).**

| Pharmacokinetic Parameters | Unit | IV administration | Topical administration |
|---|---|---|---|
| $C_{max}$ | ng/ml | 40.6 ± 4.3 | 2 ± 0.5 |
| $T_{max}$ | hr | 0.16 | 4 |
| $C_{trough}$ | ng/ml | 3 ± 0.4 | 0.3 ± 0.1 |
| $AUC_{0-24h}$ | ng.hr/ml | 220 ± 29 | 21 ± 8 |
| $AUC_{0-\infty}$ | ng.hr/ml | 254 ± 34 | 29 ± 9 |
| F | % | 100 | 11±3 |

### 2.4.2 Single topical application of Tacrolimus (0.03% ointment, 0.5mg/kg) results in significantly higher local tissue concentrations, as compared to systemic administration of the same dose.

Tacrolimus concentrations in skin, muscle, and draining lymph nodes (DLNs) at 24 hours after single application of TAC (0.03 % ointment, 0.5mg/kg) or IV bolus (0.5mg/kg) is presented in **Figure 7**. TAC concentrations in skin and muscle after single application of TAC ointment were significantly higher than the values obtained after systemic administration of the same dose ($p<0.05$), which indicates the local drug delivery to the tissues at the site of application after topical drug administration. After systemic administration, TAC distributed in to the systemic circulation and the peripheral tissues.

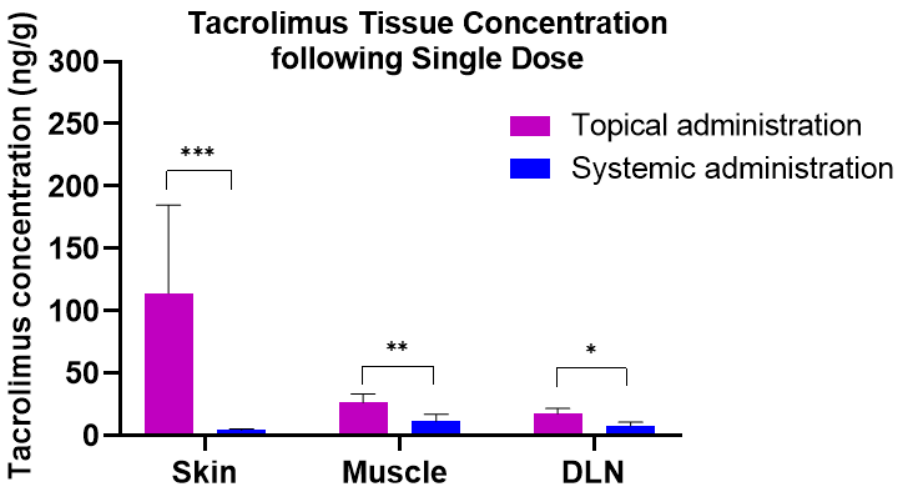



**Figure 7. Tacrolimus concentrations (ng/g) in skin, muscle, and DLNs at 24 hours after single application of tacrolimus (0.03 % ointment, 0.5mg/kg) or IV bolus (0.5mg/kg). Asterisks indicate statistical significance. *p<0.05, **p<0.01, ***p<0.01 significantly different from IV administration. Data presented as mean ± SD, n=6. P values were calculated by Mann Whitney test.**

### 2.4.3 Single topical application of Tacrolimus (0.03% ointment, 0.5mg/kg) results in significantly higher local tissue concentrations, as compared to the concentrations at the contralateral sites.

Tacrolimus concentrations in the skin, muscle, and DLNs collected from the application limbs and contralateral limbs following a single application of TAC (0.03% ointment, 0.5mg/kg) are presented in **Figure 8**. TAC concentrations in skin, muscle, and DLNs collected from the application limbs are significantly higher than TAC concentrations in the skin, muscle, and DLNs collected from the other contralateral limbs ($p<0.05$). Tissue to blood concentration ratios at 24 hours following a single application of TAC (0.03% ointment, 0.5mg/kg) or IV bolus of the same dose is presented in **Table 7**. The high tissue to blood concentration ratios indicates the direct permeation and accumulation of TAC into the local tissues including skin, muscle, and DLNs at the site of application after topical delivery. The tissue to blood ratios were significantly higher than the values observed after IV dose. This result thus indicates the possibility of targeting drugs by topical delivery to specific tissues without resulting in high blood levels.

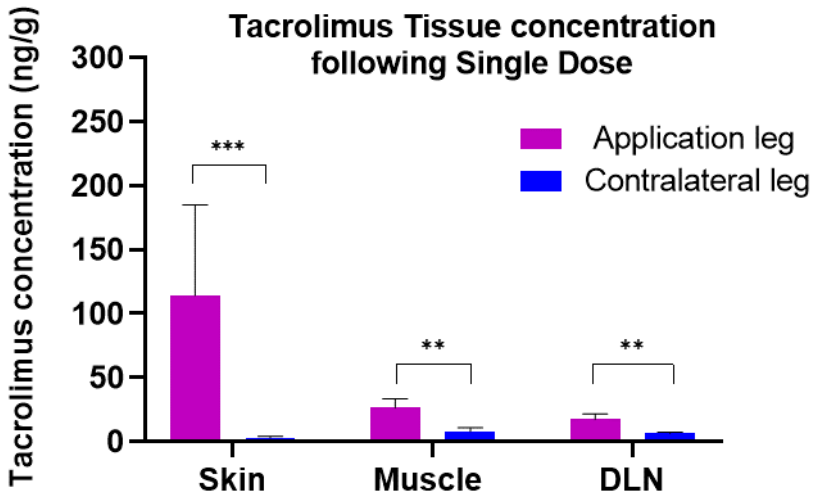



**Figure 8. Tacrolimus concentrations (ng/g) in skin, muscle, and DLNs collected from the application leg and contralateral leg at 24 hours following single application of tacrolimus (0.03% ointment, 0.5mg/kg). Asterisks indicate statistical significance. **p<0.01, ***p<0.001, significantly different from the contralateral leg. Data is presented as mean ± SD, n=6. P values were calculated by Wilcoxon Matched pairs test.**

**Table 7. Tissue to blood concentration ratios at 24 hours following single application of tacrolimus (0.03 % ointment, 0.5mg/kg) or IV bolus (0.5mg/kg)**

| Tissue-blood concentration ratios | Systemic Administration | Topical Administration (Application Site) | Topical Administration (Contralateral Site) |
|---|---|---|---|
| Skin-blood concentration ratio | 2 | 303 | 7 |
| Muscle-blood concentration ratio | 4.6 | 73 | 19 |
| DLNs-blood concentration ratio | 3.2 | 48 | 16 |

### 2.4.4 Time course of tacrolimus concentrations in skin and muscle over 24 hours after single topical application of tacrolimus (0.03% ointment, 0.5mg/kg)

The time course of TAC concentrations in the skin and muscle at 2, 6, 12, and 24 hours following single application of TAC (0.03% ointment, 0.5mg/kg) is shown in **Figure 9**. Pharmacokinetic parameters of TAC in the skin and muscle following a single application of TAC (0.03% ointment, 0.5mg/kg) are determined and presented in **Table 8**. Peak concentrations of TAC in skin and muscle were reached 2 hours post topical dose administration (661 ± 141 and 69 ± 10 ng/g respectively), then TAC concentration gradually declined over time to reach low concentrations at 24 hours post topical dose administration (171± 51 and 26 ± 9 ng/g respectively). Peak concentrations of TAC in blood were reached 4 hours post-topical dose administration (2 ± 0.4 ng/ml), then concentrations gradually declined over time to reach low concentration at 24 hours post-topical dose administration (0.3 ± 0.1 ng/ml). Drug exposure ($AUC_{0-\alpha}$) in the skin and muscle was significantly higher than the values observed in the blood (**p=0.0075, ***p=0.0004). Drug exposure ($AUC_{0-\alpha}$) in the skin was significantly higher than the values observed in the muscle (****p<0.0001). TAC concentrations in the blood was ≤2ng/ml during this time period.

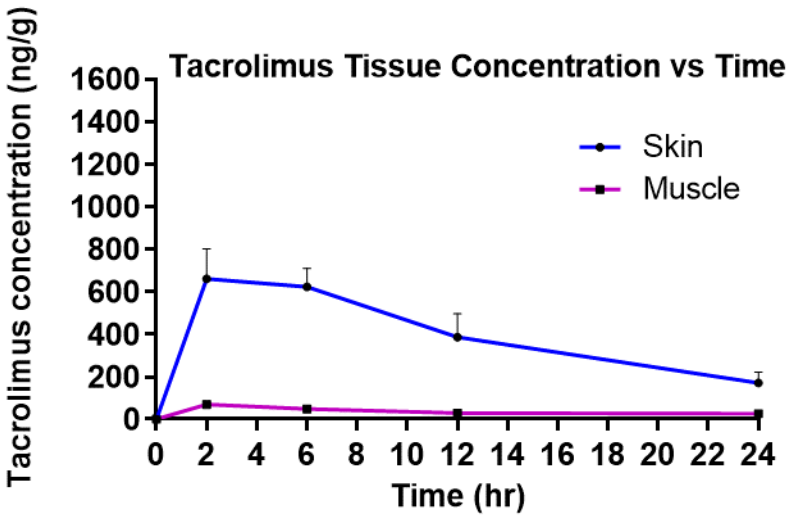



**Figure 9. Time course of tacrolimus concentrations in skin and muscle following a single application of tacrolimus ointment (0.03%, 0.5mg/kg). Data presented as mean ± SD, n=3.**

**Table 8. Comparative non-compartmental pharmacokinetic exposure parameters of tacrolimus (mean + s.d.) in the skin, muscle, and blood following single application of tacrolimus ointment (0.03%, 0.5mg/kg) (n=3).**

| | $AUC_{0-\alpha}$ (ng.hr/ml) | $C_{max}$ (ng/g or ng/ml) | $C_{trough}$ (ng/g or ng/ml) |
|---|---|---|---|
| Skin | 12497 ± 2930 | 661 ± 141 | 171 ± 51 |
| Muscle | 1567 ± 449 | 69 ± 10 | 26 ± 9 |
| Blood | 29 ± 9 | 2 ± 0.4 | 0.3 ± 0.1 |
| Skin-blood ratio | 520 | 1249 | 428 |
| Muscle-blood ratio | 65 | 129 | 60 |

### 2.4.5 Average daily trough TAC concentrations in blood after topical application (0.03% ointment, 0.5mg/kg) was significantly lower than the concentrations observed after systemic administration of the same dose

Average daily trough levels of TAC in the blood of animals received daily topical applications of TAC (0.03 % ointment, 0.5mg/kg) for 7 days was below than 3 ng/ml (sub-therapeutic). These levels are significantly lower than the values (range: 4.7-6.3ng/ml) obtained in the animals received daily intraperitoneal injections (0.5mg/kg) for 7 days (**p = 0.0012) as shown in **Figure 10**. This indicates systemic exposure was reduced with topical administration.

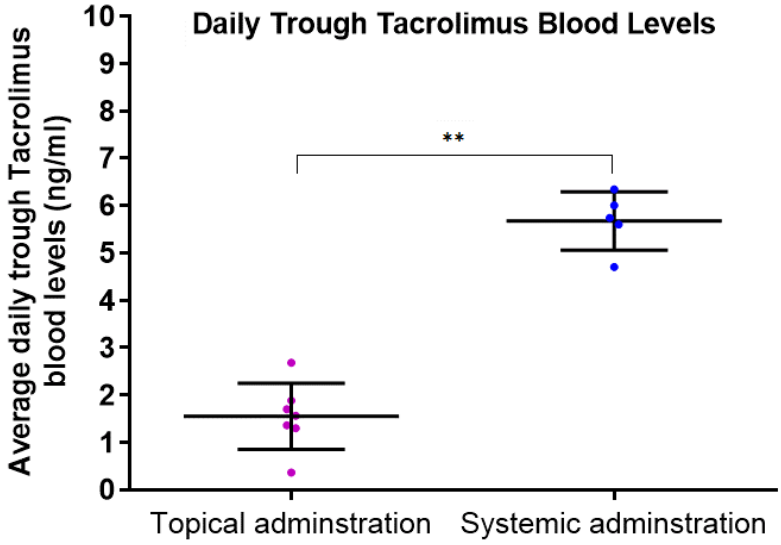



**Figure 10. Average daily trough tacrolimus concentrations in blood following daily topical application of tacrolimus (0.03% ointment, 0.5mg/kg) or IP dose (0.5mg/kg) for 7 days. Data presented as mean ± SD, n=5/topical group, n=6/systemic group. Asterisks indicate statistical significance. **p<0.01, significantly different from the systemic TAC therapy. P values were calculated by Student t test.**

### 2.4.6 Daily topical application of TAC (0.03% ointment, 0.5mg/kg) for 7 days results in significantly higher local tissue concentrations and lower blood levels as compared to the concentrations after daily systemic administration of same dose

As shown in **Figure 11**, TAC concentrations in blood, skin, muscle, and DLNs collected from the application leg at 24 hours after the last ($7^{th}$) topical application of TAC (0.03 % ointment, 0.5mg/kg) were significantly higher than the values observed after systemic administration of the same dose ($p<0.05$), while TAC concentrations in the blood were significantly lower than the values observed after systemic administration ($p<0.001$). This indicates the low systemic drug accumulation and exposure after repeated topical applications of TAC. Tissue to blood concentration ratios at 24 hours after the last ($7^{th}$) topical application of tacrolimus ointment (0.03%, 0.5mg/kg) or IP injection (0.5mg/kg) following daily topical applications or IP injections of tacrolimus for 7 days is presented in **Table 9**. The high tissue to blood concentration ratios indicates the accumulation of TAC into the local tissues including skin, muscle, and DLNs after repeated topical application. The tissue to blood concentration ratios were significantly higher than the values observed after intraperitoneal doses. This result thus indicates the possibility of targeting drugs by topical delivery to specific tissues without resulting in high blood levels.

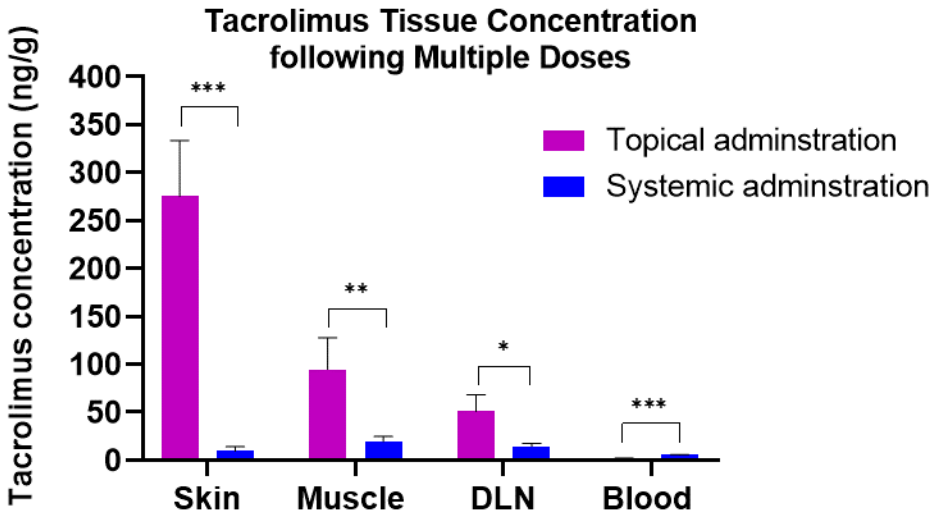



**Figure 11. Average Tacrolimus concentrations (ng/g or ng/ml) in skin, muscle, DLN, and blood at 24 hours after the last (7th) topical application of tacrolimus ointment (0.03%, 0.5mg/kg) or IP injection (0.5mg/kg). Asterisks indicate statistical significance. *p<0.05, **p<0.01, ***p<0.001, significantly different from systemic administration. Data presented as mean ± SD, n=3. P values were calculated by Student t test.**

**Table 9. Tissue to blood concentration ratios at 24 hours of the last (7th) topical application of tacrolimus ointment (0.03%, 0.5mg/kg) or IP injection (0.5mg/kg) following multiple once daily topical applications or IP injections of tacrolimus for 7 days.**

| Tissue to blood concentration ratio | Systemic Administration | Topical Administration (Application Site) |
|---|---|---|
| Skin-blood concentration ratio | 2.5 | 368 |
| Muscle-blood concentration ratio | 5.4 | 57 |
| DLNs-blood concentration ratio | 4 | 30 |

### 2.4.7 Daily topical application of TAC (0.03% ointment, 0.5mg/kg) for 7 days results in significantly higher local tissues concentrations, as compared to the concentrations in the contralateral sites

TAC concentrations in skin, muscle, and DLNs collected from both the application limbs and the contralateral limbs at 24 hours after the last ($7^{th}$) topical application of TAC ointment (0.03%, 0.5mg/kg) are presented in **Figure 12**. TAC concentrations in skin, muscle, and DLNs collected from the application limb is much higher than TAC concentrations in the skin, muscle, and DLNs collected from the contralateral limb ($p<0.05$).

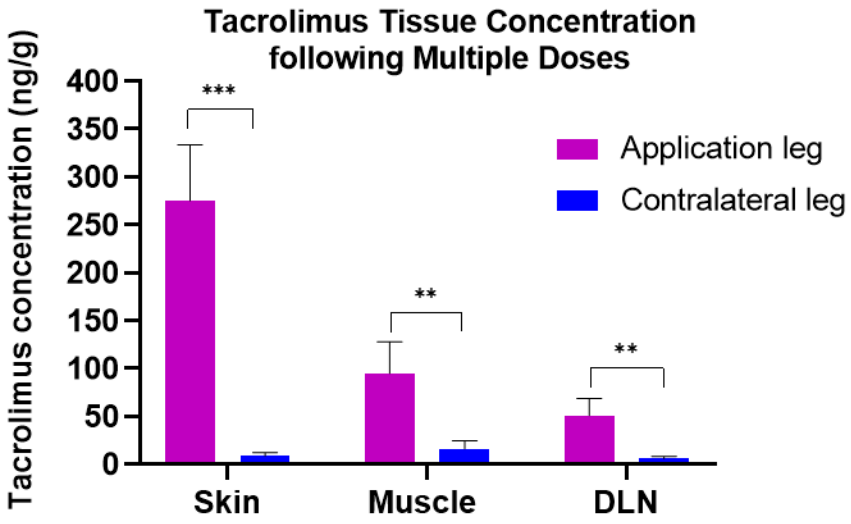



**Figure 12. Tacrolimus concentrations (ng/g) in skin, muscle, and draining lymph nodes (DLNs) collected from the application leg and contralateral leg at 24 hours after the last ($7^{th}$) topical application of tacrolimus ointment (0.03%, 0.5mg/kg). Asterisks indicate statistical significance. *p<0.05, ***p<0.001 significantly different from the contralateral leg. Data presented as mean ± SD, n=3. P values were calculated by paired t test.**

### 2.4.8 Daily topical application of TAC (0.03% ointment, 0.5mg/kg) for 7 days results in significantly higher local tissues concentrations, as compared to the concentrations observed after single topical application

TAC concentrations in skin, muscle, and DLNs collected from both the application limbs at 24 hours after the first ($1^{st}$) topical application of tacrolimus (0.03% ointment, 0.5mg/kg) or the seventh ($7^{th}$) topical application of tacrolimus ointment are shown in **Figure 13**. As results show, from day 1 ($1^{st}$ topical dose) to day 7 ($7^{th}$ topical dose), there was significant increase in the TAC concentrations in the skin, muscle, and DLNs from 114±70 ng/g, 26±7 ng/g, and 17±4 ng/g to 275±58 ng/g, 95±33 ng/g, and 51±17ng/g respectively, $^{**}p<0.01$. This indicates the local drug accumulation and exposure after repeated once daily topical applications of TAC (0.03% ointment, 0.5mg/kg).

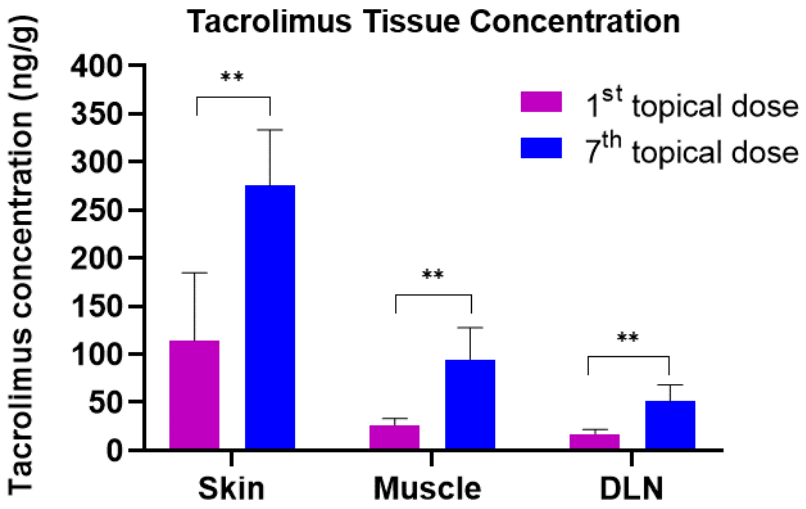



**Figure 13. Tacrolimus concentrations (ng/g) in skin, muscle, and draining lymph nodes (DLNs) collected from the application leg at 24 hours following the first topical dose or the seventh topical dose. Asterisks indicate statistical significance. **p<0.001 significantly different from the single topical application. Data presented as mean ± SD, n=3. P values were calculated by Mann Whitney test.**

### 2.4.9 Average tacrolimus concentrations in the blood over 24 hours following single topical application of tacrolimus (0.03% ointment, 0.5mg/kg) or repeated once daily topical applications.

Average tacrolimus concentrations in the blood over 24 hours following the first ($1^{st}$) topical application of tacrolimus (0.03% ointment, 0.5mg/kg) or the seventh ($7^{th}$) topical application of tacrolimus ointment are shown in **Figure 14**. The comparative non-compartmental pharmacokinetic exposure parameters of TAC in the blood following single topical dose or multiple once daily topical dose (0.5mg/kg) for 7 days of TAC are presented in **Table 10**. From day 1 to day 7, TAC accumulated to a moderate extent in blood. The mean trough concentration of TAC following the first topical dose on day 1 was 0.3 ± 0.1 ng/ml, after which the mean trough

levels increased to 1.6 ± 0.2 ng/ml on day 7 (daily topical doses). However, these levels are lower than the values observed after the first systemic dose (3 ± 0.4 ng/ml). Despite that the mean $AUC_{0\text{-}24h}$ increased from 21 ± 8 ng·h/ml on day 1 to 51 ± 7ng·h/ml on day 7, the difference was not statistically significant ($p>0.05$). The estimated ratio of accumulation from day 1 to day 7 was 2.4. However, these values are significantly lower than the values observed after single systemic dose (220 ± 29 ng·h/ml).

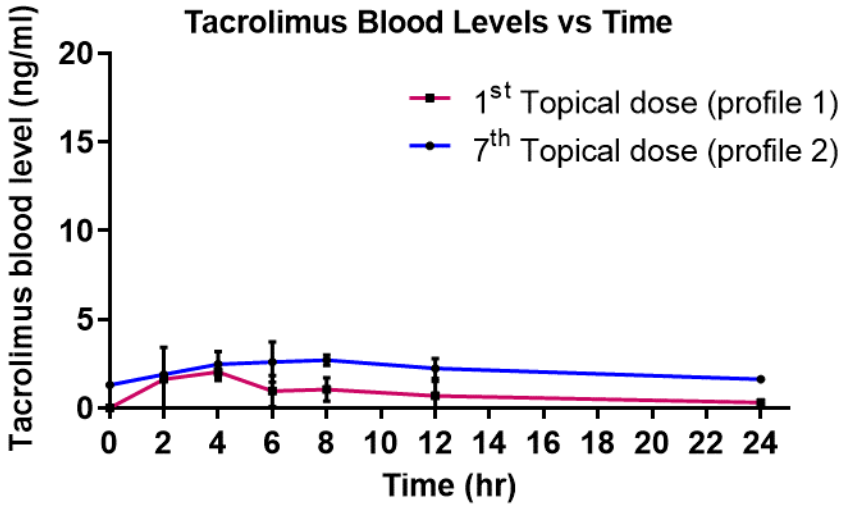



**Figure 14. Average tacrolimus concentrations in the blood over 24 hours following the first topical application of tacrolimus (0.03% ointment, 0.5mg/kg) (Profile 1) or the seventh topical applications of tacrolimus (Profile 2). Data presented as mean ± SD, n=3.**

**Table 10. Comparative non-compartmental pharmacokinetic exposure parameters of tacrolimus (mean + s.d.) in the blood following single IV dose (0.5mg/kg), single topical dose, and daily topical dose (0.5mg/kg) for 7 days of tacrolimus (Data shown as mean+SD, n=3**

| PK Parameters | Unit | 1st systemic dose | 1st topical dose | 7th topical dose |
|---|---|---|---|---|
| $C_{trough}$ | ng/ml | 3 ± 0.4 | 0.3 ± 0.1 | 1.6 ± 0.2 |
| $AUC_{0-24h}$ | ng.hr/ml | 220 ± 29 | 21 ± 8 | 51 ± 7 |
| Accumulation ratio R(ac) | | | | 2.4 |

### 2.4.10 Effect of daily topical application of TAC (0.03% ointment, 0.5mg/kg) on the body weight

The percent change in body weights of the control group (un-treated animals), systemic TAC group (0.5mg/kg/day), and topical TAC group (0.03% ointment, 0.5mg/kg/day) as compared with the initial body weights is shown in Figure 15. There were no signs of systemic toxicity in any of the animals received TAC. Animals in all groups showed significant body weight increase during the treatment periods as compared to the initial body weights ($p<0.05$). Body weight increase was similar for topical TAC group and control group in the different treatment periods ($p>0.05$). Body weight increase was smaller in the systemic TAC group as compared with the other groups during the treatment periods. However, the differences were found to be statistically non-significant ($p>0.05$).

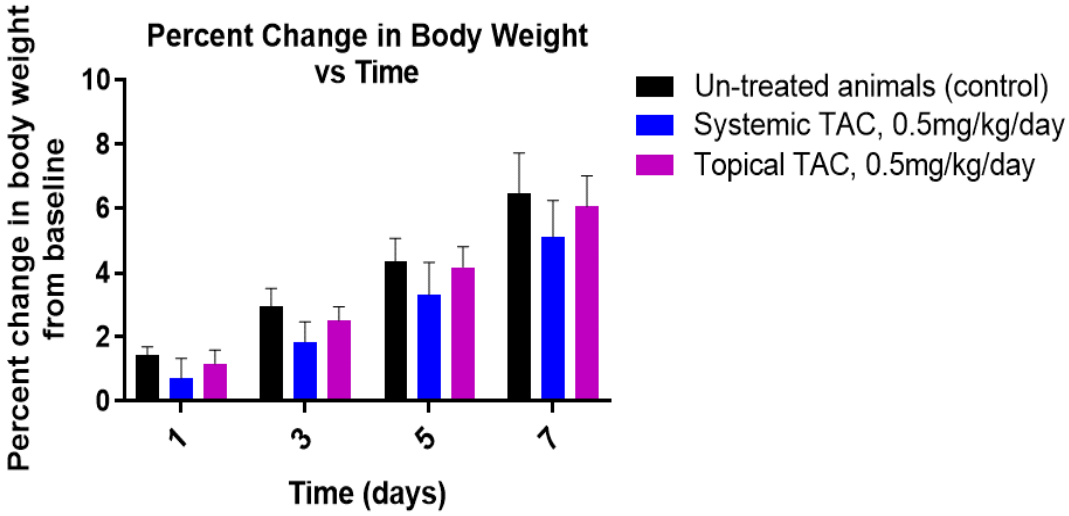



**Figure 15. Measurements of average percent change of body weight from baseline in the un-treated animals (control), and in the treated animals with systemic TAC (Intraperitoneal, 0.5mg/kg/day) or topical TAC (0.03% ointment, 0.5 mg/kg/day) during the treatment period. Data shown as mean±SD, n=3/group. P values were calculated by two-way ANOVA with Tukey's multiple comparisons test.**

## 2.5 Discussion

Skin is the most immunologically susceptible tissue in the VCA allograft and the primary target of rejection. High doses of oral TAC are administered to prevent AR episodes, but this dosing regimen is associated with serious systemic adverse effects. Topical administration of TAC could improve effectiveness of the drug by predominantly concentrating the drug in the graft, particularly skin, decreasing systemic exposure, and consequentially off-target effects. Topical immunosuppressants have been successfully used to prevent skin rejection episodes in experimental and clinical models of VCA. In an earlier work, our group reported that tacrolimus ointment (Protopic® 0.1%) prolonged allograft survival after short course systemic therapy with CsA in rat model of VCA. The concentrations of TAC were extremely high in the skin and variable in the blood. Topical tacrolimus (Protopic® 0.03%) has been also used effectively in VCA (corneal graft rejection) [142] and dermatology (atopic dermatitis) and was associated with lower systemic exposure as compared to topical tacrolimus (Protopic® 0.1%) [127-129, 143, 144].

In this study, we evaluated the ability of topical TAC (Protopic® 0.03%) at a dose of 0.5mg/kg/day to achieve high tissue concentrations at the site of application for local effects with low systemic concentrations. We assessed the pharmacokinetics and tissue distribution of topical tacrolimus (Protopic® 0.03%) following single or repeated topical application in comparison to those after systemic administration in rats. Rats with intact healthy skin were used to evaluate the systemic uptake of TAC after topical delivery. This provides information about the absorption of TAC from the topical formulation without the effect of other confounding factors that may change the permeability of TAC, systemic absorption, and exposure [145]. Briefly, the results of this study show that after topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5mg/kg, TAC was mainly delivered to the skin with limited diffusion into the blood and the other

contralateral sites. Blood levels of TAC following topical application were low and unlikely to result in systemic immunosuppression. The high tissue to blood concentration ratios indicates direct permeation of TAC into the local tissues after topical administration and indicates high affinity of TAC to the tissues. There was no topical therapy-related toxicity observed in any of the animals.

Tacrolimus exposure in the blood and the local tissues including skin, muscle, and DLNs after single topical application of TAC (Protopic® 0.03%) at a dose of 0.5mg/kg was evaluated. The results revealed that systemic exposure of TAC as measured by $AUC_{0-\infty}$, $C_{max}$, and $C_{trough}$ after topical delivery were markedly lower than the values obtained after systemic delivery of the same dose. A small percent (11%) of the topically applied dose reached the systemic circulation over the time period measured. This was probably due to the drug accumulation in the local tissues at the application site (particularly skin), followed by slow and gradual diffusion of the drug into the system. Following topical delivery, drug slowly and gradually diffuses into the epithelial layers of the skin, and later to the deeper tissues. TAC has large molecular weight (804.018 g/mol) and high lipophilicity (log P = 3.96±0.83) [146] which limits the drug's ability to pass across the skin layers and mainly retained in the lipid-rich layer 'stratum corneum' [147]. Skin, muscle, and DLNs had significantly higher TAC concentrations when compared to the blood (300, 66, and 43-fold higher). However, the skin component appeared to retain most of the drug when levels across skin, muscle, and DLNs were compared. This is desirable because skin is the target tissue for rejection in VCA.

Tacrolimus concentrations in the skin and muscle collected from the application site were significantly higher than the values observed after systemic delivery of the same dose. This indicates the local drug delivery to the tissues at the site of application after topical drug delivery.

Tissue to blood concentration ratios of the topical dose were significantly higher than tissue to blood concentration ratios of the i.v. dose. This supports the possibility of targeting drugs to local tissues by topical administration without high blood levels.

Average TAC concentration in skin, muscle, and DLNs collected from the application site was significantly higher than drug concentrations in the tissues collected from the contralateral site. This indicates that TAC mainly localizes to the site of topical application with limited distribution to other sites remote from the application site. TAC was also measurable in DLNs, and this may be related to the lipophilicity of TAC [148]. Low TAC concentrations in the tissues collected from the contralateral site reflects the low systemic absorption of TAC after topical administration.

Time course of tacrolimus concentrations in skin and muscle over 24 hours after single topical application of tacrolimus (Protopic® 0.03%) once daily at a dose of 0.5mg/kg was evaluated. TAC concentrations in skin and muscle tissues reached the highest values in the period between 2 and 6 hours post topical dose administration, which indicates the gradual uptake and diffusion of the drug into these tissues or compartments after topical administration. Then, the concentrations gradually declined to reach low concentrations at 24 hours post topical dose administration. This is due to the drug removal or clearance from the tissues by the systemic circulation. Additionally, skin (stratum corneum) retards systemic drug absorption and slow down the elimination process in the tissues. Results showed that single topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5mg/kg provides high local drug exposure at the site of application and low systemic exposure. Most of the amount applied topically was absorbed within 24 hours. Efficacy studies are needed to determine the target effective therapeutic tissue concentrations that should be achieved to prevent rejection.

Tacrolimus exposure in the local tissues and blood after repeated topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5mg/kg was also evaluated and compared to the values obtained after single topical application. Systemic exposure to TAC following repeated topical application as measured by $C_{troughs}$ and $AUC_{0-\infty, ss}$ was higher than the values obtained after single topical application (2-fold higher) indicating moderate systemic accumulation of TAC. However, systemic exposure to TAC following repeated topical applications was significantly lower than the value observed following systemic administration of the same dose. This indicates that the systemic exposure of TAC was low with topical administration despite the repeated applications. Average TAC concentrations in the local tissues were significantly higher than the values obtained after single topical delivery, indicating the local accumulation of TAC after repeated topical applications. The dosing regimen can be further modified to minimize the systemic or local TAC accumulation after repeated applications.

However, it is important to determine whether the concentrations of TAC observed in the blood does not result in systemic immunosuppression, and the concentrations of TAC observed in the local tissues particularly skin are sufficient to exert a therapeutic effect. The observed blood and tissues concentrations should be compared with the minimal concentrations of TAC that have been reported to be effective. Despite that the lowest trough TAC blood levels at which systemic immunosuppressive effects can be observed is not known, studies showed that trough blood TAC levels needed to prevent rejection are between 5-10 ng/ml [61, 149], while lower trough blood levels (<5ng/ml) could result in allograft rejection and thus considered “sub-therapeutic” [150]. Studies reported that doses between 0.5-1mg/kg (systemic TAC) are sufficient to achieve the therapeutic blood levels of TAC and has shown efficacy in preventing rejection in solid organ and limb transplantation [151]. Un-published data from our laboratory showed indefinite allograft

(hind limb) survival of animals receiving daily intraperitoneal injection of TAC in a dose of 0.5mg/kg.

Our results showed that systemic administration of TAC at dose of 0.5mg/kg/day provides trough blood TAC levels ranged between 4.7-6.3ng/ml. These are the minimum effective therapeutic levels that should be achieved in the blood to prevent rejection. Our results revealed that single topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5mg/kg results in lower concentrations of TAC in the blood and higher concentrations in the skin, muscle, and DLNs, when compared to the concentrations observed after systemic delivery of the same dose (0.5mg/kg/day). Further studies should be performed to evaluate the efficacy of TAC (Protopic® 0.03%, 0.5mg/kg/day) in preventing skin rejection in a rat model of VCA.

In animal studies, systemic administration of TAC has been associated with systemic toxicity including metabolic complications [152, 153]. Systemic TAC decreases the body weight gain rate over time [154]. Here, we evaluated the impact of topical application of TAC (Protopic® 0.03%) once daily at a dose of 0.5mg/kg on body weight change from baseline. Animals received topical TAC showed significant body weight increase during the treatment periods as compared to the initial body weights. Body weight increase was smaller in the systemic TAC group (0.5mg/kg) during the treatment periods as compared with topical TAC group suggesting that the difference can be attributed to increased systemic exposure to TAC after systemic administration. Body weight increase was similar for topical TAC group and control group indicating that systemic exposure to TAC after topical application is low. TAC ointment (Protopic® 0.03%) is well tolerated effective formulation for local delivery of TAC in VCA.

# 3.0 Mycophenolic Acid for Topical Immunosuppression in Vascularized Composite Allotransplantation: Optimizing Formulation and Evaluation of Pharmacokinetics and Tissue Distribution

## 3.1 Abstract

Mycophenolic acid (MPA), the active form of the ester prodrug mycophenolic mofetil (MMF), is an FDA approved immunosuppressant. MMF has been successfully used in combination systemic therapy with other immunosuppressants for the prevention of AR episodes following SOT and VCA. Systemic use of MMF is associated with gastrointestinal adverse effects. Topical delivery of the active drug could provide site-specific immunosuppression while minimizing systemic toxicity. Limited data is available on topical MPA in experimental setting in dermatology. MPA has not been systematically evaluated for use in VCA as topical treatment. Our goal was to develop a topical formulation of MPA with optimal in vitro / in vivo characteristics such as release, permeation, and tissue bioavailability to enable safety and efficacy evaluation in clinical VCA. In-vitro permeation studies were performed with a solution of MPA (10mg/ml), and with different semisolid formulations of MPA (1%w/w) (Aladerm, Lipoderm, Emollient, and Versa Base) using a Franz Diffusion Cell System (FDCS). In-vivo pharmacokinetics and tissue distribution of MPA in Lipoderm were evaluated in rats. Of the four semisolid formulations tested, a moderate amount of MPA (1%w/w) permeated the biomimetic membranes by the 24-h time point with Lipoderm formulation. The timeline and kinetics of drug diffusion and release were optimal with MPA in Lipoderm. This included gradual diffusion and sustained drug release which facilitates a prolonged local site-specific action of the drug. The cumulative release of MPA from Lipoderm, showed a linear single-phase profile with a $R^2$ of 0.969, flux ($1.12 \pm 0.24$ μg/cm$^2$/hr), and permeability ($6.2 \pm 1.3 \times 10^{-8}$cm/s). The formulation was stable over 6 months of storage at 25 °C. In-vivo, MPA in Lipoderm (1%w/w) showed significantly higher local tissue concentrations and lower systemic exposure as compared to values observed after systemic administration ($p<0.05$). We successfully developed, a topical formulation of MPA (Lipoderm 1%) with good in

vitro/in vivo characteristics and without local adverse effects. Our study provides key preliminary groundwork for translational efficacy studies of topical MPA in pre-clinical animal VCA models and for evaluation of effectiveness in patients receiving VCA.

[Feturi, Firuz G et al. *"Mycophenolic Acid for Topical Immunosuppression in Vascularized Composite Allotransplantation: Optimizing Formulation and Preliminary Evaluation of Bioavailability and Pharmacokinetics."* Frontiers in surgery vol. 5 20. 9 May. 2018]

## 3.2 Introduction

Currently, TAC (Protopic™ ointment 0.1%, 0.03%, Astellas), and Clobetasol (Temovate ® ointment, cream 0.05%, GlaxoSmithKline), are FDA approved for topical use in certain dermatological conditions [155-159]. These topical immunosuppressants have been used in VCA, off-label, to treat AR episodes pro re nata (PRN) [150]. With the exception of TAC and clobetasol, there are no commercially available topical formulations for other widely used systemic immunosuppressants such as MPA, rapamycin (RAPA), and everolimus. It is therefore important to investigate the feasibility of developing other topical immunosuppressant formulations with independent or synergistic efficacy and safety profiles. Developing an optimal topical formulation of MPA addresses this timely clinical need in VCA.

MPA is the active form of MMF, an immunosuppressant used in SOT. Unlike calcineurin inhibitors like CsA and TAC, MPA is not associated with organ toxicity, malignancy, and cardiovascular complications [160-164]. Over the past two decades, MPA has been used in triple therapy regimens in combination with TAC and prednisone in SOT [111, 165] or VCA [56, 112]. It has also been used in dual therapy in combination with RAPA in VCA [166, 167]. MPA is commercially available as the ester prodrug MMF (CellCept®, Roche), or as mycophenolate sodium (Myfortic®, Novartis) in an enteric-coated form [168]. In vivo, the prodrug MMF is converted via hydrolysis by carboxylesterases to MPA [94]. This conversion occurs in blood, liver, kidneys and to a small extent in skin [104]. MPA is metabolized by UDP- glucuronyl transferase to form MPA glucuronide (MPAG), which is an inactive metabolite that is excreted in the urine and bile as shown in **Figure 16** [169]. In addition to its immunosuppressive effects, MPA has antibacterial, antifungal, and antiviral properties [170-172]. MPA exerts its effects on T and B cells by reversible inhibition of inosine monophosphate dehydrogenase (IMPDH), an enzyme

essential in the de-novo-synthesis of guanosine nucleotides required for DNA and RNA synthesis [173, 174]. Despite its therapeutic efficacy, systemic use of MPA/MMF has been associated with gastrointestinal (nausea, diarrhea, abdominal cramps, constipation) and genitourinary (urgency, frequency, dysuria, hematuria) adverse effects [88, 110, 175].

Topical administration of MPA in VCA could facilitate minimization of overall number of drugs, dose, dosing frequency of systemic immunosuppressants while improving its antirejection efficacy and effectiveness in graft survival [94, 176]. Furthermore, such strategies could allow graft targeted delivery with predominantly localized action, reduce risk of systemic side effects, avoid first pass intestinal/hepatic metabolism, combine multiple drugs and potentially minimize drug-drug interactions [94, 106]. Limited data is available on topical MPA/MMF in experimental setting in dermatology. It has not been systemically evaluated for VCA as topical treatment.

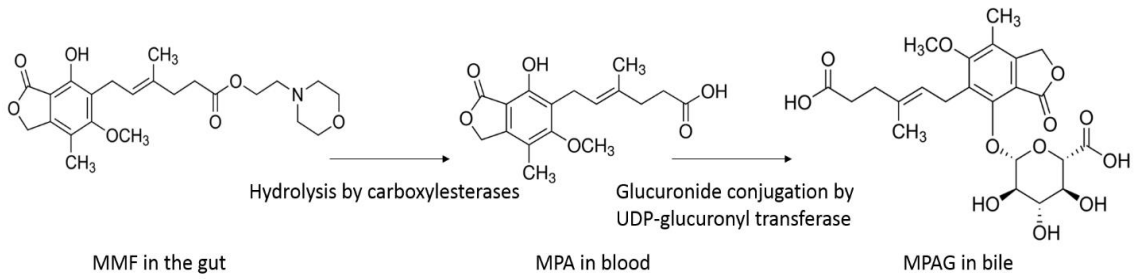



**Figure 16. Pathway of mycophenolic mofetil in the body.**

For efficacy, any topical drug formulation must first consider the challenging barrier of the stratum corneum in the skin [177-180]. Compounds like MPA with low molecular weight (320.33g/mol), moderate lipophilicity (logP 3.8), and low acidity (pKa 3.5) offer superior skin penetration and permeation to the underlying tissues and thereby suitable candidate for topical delivery [162, 181]. However, in-vitro and in-vivo studies are performed to evaluate the ability of

the topical formulation to penetrate the skin and permeate to the target areas. We hypothesized that topical delivery of MPA will provide high concentrations at the site of application for local effect while decreasing systemic exposure and consequentially off-target effects. The goal of this study was to prepare a topical formulation of MPA with good in vitro / in vivo characteristics such as release, permeation, and tissue bioavailability for further safety and efficacy evaluation in clinical VCA.

## 3.3 Materials and Methods

### 3.3.1 Chemicals and Reagents

The chemical structures of MPA and the internal standard, MPA-D3 are represented in **Figure 17**. MPA powder was obtained from Sigma–Aldrich (St. Louis, MO, USA). Deuterated mycophenolic acid (MPA-D3) was purchased from Toronto Research Chemicals (TRC): Cat No: M831502. Cremophor (kolliphore®), and propylene glycol USP were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lipoderm, Aladerm, Versabase and Emollient cream were manufactured by PCCA (Professional Compounding Centers of America). Semisolid formulations of MPA aladerm, MPA lipoderm, MPA emollient, and MPA versa base were compounded by Hieber's Pharmacy (Pittsburgh, PA, USA). Spectra/pro RC membrane discs, MWCO: 6-8000 Dalton, thickness 0.002 inches, were purchased from spectrum chemicals ® (Rancho Dominguez, CA, USA). All the solvents were HPLC and MS grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sigmacote® siliconizing reagent for glass was obtained from Sigma–Aldrich (St. Louis, MO, USA).

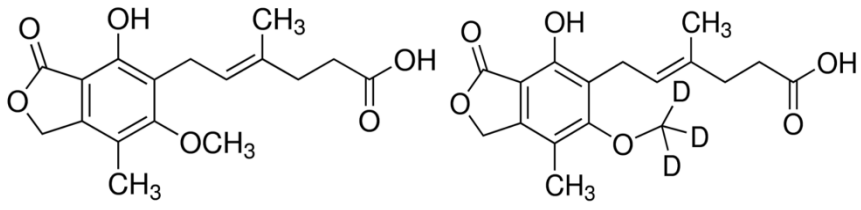



**Figure 17. Chemical structures of MPA (right, molecular weight: 320.33g/mol) and MPA-D3 (left, internal standard, molecular weight: 323.36 g/mol).**

### 3.3.1 Animals

All experiments were performed in accordance with a protocol reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Animals (inbred Male Lewis rats aged 8 to 10 weeks, weighing about 300 to 320 g at the time of study dose, Charles River Laboratories, Horsham, PA), were housed in a specific pathogen-free barrier facility and maintained in accordance with IACUC guidelines. All procedures were in compliance with American Association for the Accreditation of Laboratory Animal Care (AALAC) recommendations and the principles set forth in the National Institute of Health Publication 80-23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended. Animals were housed individually and with plastic Elizabethan collars to prevent oral ingestion of the topical formulations, and to prevent access of animal to the drug application site.

### 3.3.2 Assessment of Partition Coefficient of Mycophenolic Acid in Octanol/Water

Partition coefficient of MPA was experimentally measured to evaluate the partitioning ability of MPA into the VCA graft. One milligram of MPA powder was put in 2-mL tubes and sealed. One milliliter of 1-octanol and 1 mL of potassium phosphate buffer (pH=7.4) was added to the MPA powder. After vortexing for 5 min and ultra-sonication at 25°C for 15 min, samples were centrifuged for 5 min at 13,400 rpm to facilitate phase separation and allowed to stand for 1 hour. The octanol was separated from the aqueous phase. Samples were diluted with acetonitrile and analyzed by high-performance liquid chromatography (HPLC). The partition coefficient (log Po/w) as a measure of lipophilicity was calculated as follows: $log\ P_{o/w} = log\ (C_o/C_w)$, $C_o$ and $C_w$

are the concentrations of MPA in the octanol and in the water phase, respectively as per standard methods.

### 3.3.3 Preparation of Semisolid Formulations

Semisolid formulations for MPA were compounded at Hieber's Pharmacy (Pittsburgh, PA, USA) using the following ingredients: MPA (Active ingredient), Propylene Glycol USP (solubilizing agent/penetration enhancer), and water, Isopropyl Myristate, Phospholipids, Cetearyl Alcohol Triticum Vulgare (Wheat) Germ Oil, Cetyl alcohol, Stearyl alcohol, Ceteareth-20, Caprylic/Capric, Triglycerides, Glycerin, Dimethicone C13-14 Isoparaffin, Laureth-7, Xanthan Gum, Magnesium Aluminum Silicate Polyacrylamide, Disodium EDTA, BHT, Phenoxyethanol, Methylchloroisothiazolinone, and Methylisothiazolinone (Base/excipients). The prepared formulation was visually examined followed by light microscopy. All formulations were stored in at room temperature (25±2° C).

### 3.3.4 Morphology of Semisolid Mycophenolic Acid Formulation:

The prepared topical formulations were visually examined for its appearance, color, and presence of aggregates or lumps. Light microscopy was used to examine the appearance of the semisolid formulation and the distribution of the particles within the formulation, and to ensure the absence of large clumps. The texture or consistency was examined by rubbing a small amount of the formulation on the skin and observing for absence of grittiness. The pH was measured in aqueous solutions of the formulation, using a digital pH meter.

### 3.3.5 In Vitro Permeation Study of Mycophenolic Acid in Solution:

Permeation (partitioning and diffusion) of MPA in solution across artificial skin was simulated in a Franz diffusion cell system (PermeGear, Nazareth, PA). Donor compartment separated from the recipient compartment by a pre-hydrated biomimetic semi-permeable membrane (Spectra/Por® RC Membrane Discs (molecular weight cutoff, 6,000 to 8,000; Spectrum Chemical, Gardena, CA) as shown in **Figure 18**. MPA solution was prepared in a combination of cremophor (15%), ethanol (10%), and deionized water. Donor compartment was loaded with 400 µl of MPA solution (10 mg/ml) using a positive-displacement pipette. The effective diffusion area was 1.77 $cm^2$. A similar vehicle was used in the receptor compartment. The receptor medium was stirred using a magnetic stirrer and maintained at 32 ± 0.1°C to reflect the normal skin temperature. The amount of drug permeated through the membrane was determined by collecting aliquots from receptor compartment at 0, 1, 2, 4, 6 and 24 hr and replaced with the same volume of fresh receptor medium. The samples were analyzed by HPLC assay method. All experiments were performed in triplicates. The cumulative amount of MPA diffused over a period of 24 hr was plotted against time.

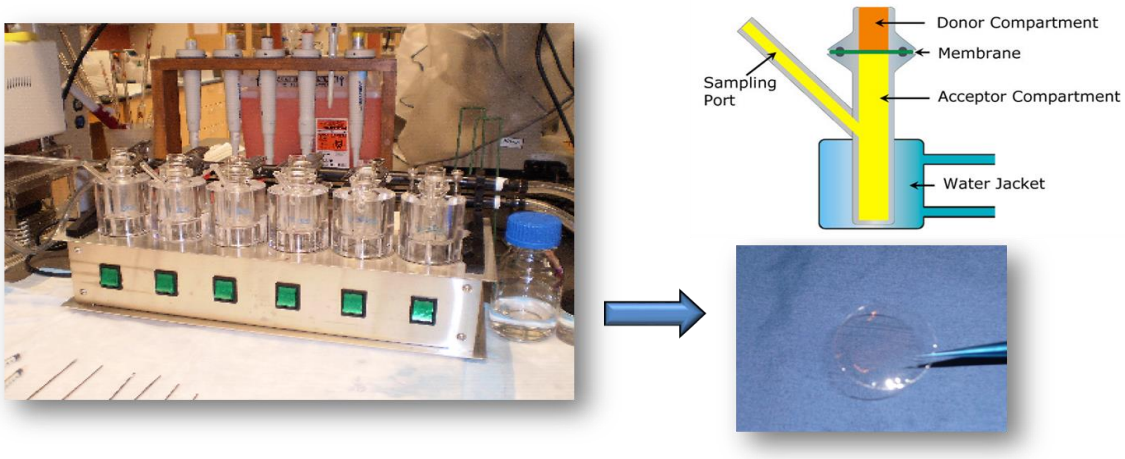



**Figure 18. Franz diffusion cells system.**

### 3.3.6 In Vitro Release and Permeation Studies for Mycophenolic Acid from Different Semisolid Formulations:

Release and permeation of MPA from four different semisolid formulations was evaluated in a Franz diffusion cell system. A drug containing formulation (1%w/w, 0.5g) was applied in the donor compartment. The aqueous receptor medium was stirred using the hotplate magnetic stirrer maintaining the temperature at 32 ± 0.1$C^0$. The amount of drug permeated through the membrane was determined by collecting aliquots from the receptor compartment at 0, 1, 2, 4, 6, 12, and 24hr and replaced with the same volume of fresh receptor solution. The drug in the receptor compartment was analyzed by validated HPLC method (as described below). The cumulative amount of drug diffused over a period of 24 hr was plotted against time. The permeation coefficient ($K_p$) of MPA was calculated using equation derived from the Fick's First Diffusion Law; $J_{ss}=K_p$ x C. $J_{ss}$ is the steady-state diffusion flux (slope of the linear portion of cumulative amount permeated vs. time profile (µg/s) / area exposed ($cm^2$)), and $C_s$ is the saturated drug concentration at the donor compartment (µg/ml).

### 3.3.7 Stability test

Stability tests were performed to evaluate the effect of the storage condition on the formulation and to determine the shelf life. These tests were performed on samples kept for a period of two and six months at 25±2 °C. Color, phase separation, and liquefaction were evaluated, and percent of MPA in lipoderm were measured immediately after compounding, and at 2 and 6 months by HPLC-MS/MS.

### 3.3.8 In Vivo Topical Administration of Mycophenolic Acid:

The experiment was performed to evaluate drug exposure in the local tissues and the blood after single topical application of MPA. Male Lewis rats received either a single topical dose of MPA in Lipoderm (1%, 16.6mg/kg), [n=6], or IV bolus dose (10mg/kg), [n=8]. The topical formulation was applied on the right or left hind limb of the rat. Tail vein blood sampling was performed at 0.08, 0.16, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hr. Blood samples were centrifuged at 2100 ± 100 rpm for 10 min, at room temperature and the plasma was separated and stored frozen at -80 $^{0}$C until analysis. Animals were euthanized after 24 hours following MPA treatment, and tissues (skin, muscle, and DLNs) were collected from both the application limb and contralateral limb for drug level measurement. In another experiment, the local drug distribution in skin and muscle over 24 hours after topical application of MPA was evaluated. Animals (n=3) received a single topical dose of MPA in Lipoderm (1%) applied at an effective dose of 16.6 mg/kg. Biopsies from skin and muscle were collected at 2, 6, 12, and 24 hours post dose administration for measurement of tissue concentration. Before excising tissue samples, the skin was wiped with ethanol-soaked gauze. Plasma and tissue concentrations of MPA were analyzed by LC-MS/MS method.

### 3.3.9 Quantitation of Mycophenolic Acid in Diffusion Medium from In-Vitro Study was Measured using High Pressure Liquid Chromatography (HPLC).

Five hundred microliters of methanol were added to 50µl of sample solution (MPA in medium). Samples were centrifuged for 3 minutes at 13000 rpm after vortexing for 2 minutes at 3000 rpm, and the supernatants were analyzed with an HPLC assay developed and validated for

determination of MPA in a medium made up from a mixture of cremophor 15%: ethanol 10%: de-ionized water. Separation was performed by a reversed phase SYMMETRY C18 column (100Å, 5 µm, 4.6 X 250 mm) using Water Alliance System 2695-2998 with UV detection at 254 nm. Isocratic elution was performed with a mobile phase consisting of 30% water, 70% methanol, 0.1% formic acid (pH=3), flow rate of 1 ml/min, injection volume 50µl, column temperature 50 $^{o}$C. MPA had a retention time of 4.2 min. The method was selective and reproducible in the range of 0.2-10 µg/ml with $r^2$ of .9996. The lower limit of quantification (LLQ) was 0.2µg/ml. The intraday and interday CV% at 0.5, 2.5 and 5 µg/ml were less than 10% (n=3).

### 3.3.10 Quantification of Mycophenolic Acid in Plasma was performed by HPLC-Tandem Mass Spectrometry (HPLC-MS/MS).

Fifty microliters of plasma containing an unknown concentration of MPA was added to a conical centrifugation tube, followed by two hundred microliters of a solution of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$). Five hundred microliter of an acetonitrile-based solution containing a deuterated internal standard (MPA-$D_3$) at a concentration of 250 ng/L were then added and the mixture was vortexed at 3000 rpm for 2 minutes. Samples then underwent centrifugation at 13,000 rpm for 3 minutes with the supernatant being poured off and collected into individual glass LCMS vials for analysis. An identical method was employed in the preparation of the calibration curve and quality control samples. Analysis was performed using a validated, reverse phased method for the detection of MPA in plasma on a Waters Micromass Quattro micro™ API mass spectrometer in positive electrospray ionization mode, utilizing multiple reaction monitoring, with an injection volume of 20 µL of sample. The Waters 2795 Separations Module was equipped with a Atlantis dC18 column (2.1 x 20 mm, 5 µm) heated to 40° C. Analytes were effectively separated using a

gradient elution consisting of an aqueous mobile phase (95% $H_2O$ / 5% MeOH) and an organic mobile phase (100% MeOH), at a flow rate of 0.4 mL per minute. In order to optimize atomization and enhance the quality of chromatographic output, both mobile phases also contained 0.1% formic acid and 2mM ammonium acetate. Monitored parent to product mass transitions for MPA and MPA-$D_3$ were 338.2 → 207.2 and 341.2 → 210.2 m/z, respectively. Under these conditions, MPA had a retention time of 7.0 minutes. Results were shown to be linear for concentrations ranging from the limit of quantification (LoQ) value of 0.3 µg/ml up to concentration values as high as 15 µg/ml, with an $R^2$ value of 0.9996. Both intra- and inter-day precision were shown to be acceptable (C.V. <10% n=3) at concentrations of 0.5, 5, and 10 µg/mL [182].

### 3.3.11 Quantification of Mycophenolic Acid in Tissues

The skin sites for tissue sampling were wiped down three times with ethanol-soaked gauze to remove residual ointment on the surface. Skin and muscle were frozen with liquid nitrogen and pulverized in pestle and mortor to fragment the frozen tissues samples into fine pieces. Tissues were weighted and homogenized with cold methanol (1ml) in homogenization tubes using Mini-BeadBeater-1 (Cole-Parmer North America) for cell disruption. The homogenate was sonicated for 1 hour at 25 °C and then kept overnight at 4°C to allow for the complete extraction of the drug from the tissues. The homogenate was transferred to an appropriately labeled micro centrifuge tube and centrifuged at 2100 ± 100 rpm for 10 min. The supernatant was transferred to a labeled glass vial and evaporated by sample concentrator and the drug residue was reconstituted with plasma (1ml). Tissue drug concentrations are expressed as µg of MPA/g of tissue weight. To control for residual MPA Lipoderm on the skin, ointment was applied on limbs (n=4) and immediately cleaned with ethanol-soaked gauze. Biopsies from skin were collected and analyzed

for MPA concentration. The highest MPA concentrations from residual Lipoderm that remained on the skin after wiping off were minimal (0.8±0.4 µg/g) compared to the actual tissue concentrations.

### 3.3.12 Pharmacokinetic Analysis

Descriptive pharmacokinetic parameters for MPA after topical and/or systemic administration were estimated by non-compartmental analysis Phoenix WinNonlin® 6.1 (Certara, St. Louis, MO). The following exposure parameters were obtained directly from the concentration-time profiles: Maximum plasma level ($C_{max}$), and trough plasma level ($C_{trough}$), area under the plasma concentration-time curve to the final sampling point ($AUC_{0-t}$). The bioavailability of MPA after topical administration was obtained as F = ($AUC_{0-\infty}$ topical). (i.v. dose)/($AUC_{0-\infty}$ i.v.).(Topical dose). Tissue to plasma concentration ratio was obtained as tissue concentration/plasma concentration. The accumulation ratio R(ac) was obtained as $AUC_{0-t,\ ss}$ at steady state / $AUC_{0-t,\ 1}$ after the first dose. The cut off values for non-, weak, moderate, and strong accumulation can be set at $R(ac) < 1.2$, $1.2 \leq R(ac) < 2$, $2 \leq R(ac) < 5$, and $R(ac) \geq 5$, respectively.

### 3.3.13 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 statistical software for windows (GraphPad Software, La Jolla, CA, USA). Data sets were checked for normality. Student t test, Mann Whitney test, or Wilcoxon Matched pairs test was used for two groups, and analysis of variance (ANOVA) was used when one independent variable with greater than two conditions or treatments and outcomes was evaluated and compared. Post hoc test (Tukey) was used to do

multiple comparisons. All experimental results were expressed as the mean ± standard deviation (SD). A p value $< 0.05$ was considered as statistically significant difference. Statistically significant data were presented as follows: *P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. Statistical tests are specifically indicated under each figure.

## 3.4 RESULTS

### 3.4.1 In-vitro permeation studies for mycophenolic acid in solution

The cumulative amount of MPA permeated per unit area 1.77cm$^2$ from MPA solution (4mg) across membrane over 24 hours is shown in **Figure 19**. MPA in solution exhibited a steady state diffusion flux ($J_{ss}$) 3.8 ± 0.1 µg/1.77cm$^2$/hr with coefficient of variation (CV) of flux 3%. Permeation coefficient ($K_p$) of MPA across the membrane was $1.1x10^{-7}$± $3.2x10^{-9}$ cm/s. A cumulative release amount of MPA in solution plotted against the time, showed a linear profile with $R^2$ of 0.969. Total MPA amount permeated at 24 hr was 162 ± 4.6 µg. 4% of the loaded MPA dose permeated over 24 hr into the receptor chamber.

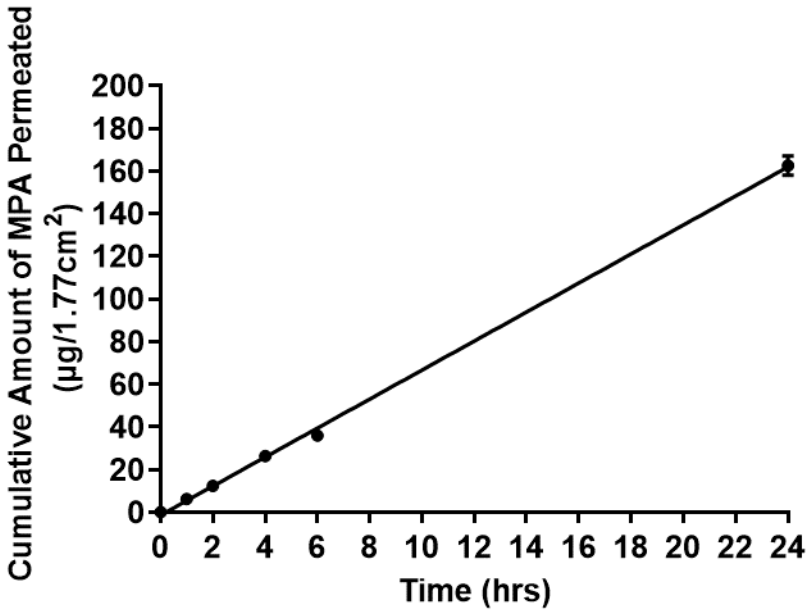



**Figure 19. Cumulative amount of MPA (µg/1.77cm$^2$) permeated across biomimetic semi-permeable membrane from MPA solution vs. Time. Data shown as mean±SD, n=3.**

### 3.4.2 Permeability and drug characteristics

The partition coefficient (log $P_{ow}$) for MPA was experimentally measured as 3.5 ± 0.1, while the predicted value was 3.8 (Drug Bank). The physiochemical characteristics of MPA compared with other immunosuppressive drugs (TAC and Rapa) are presented in **Table 11.** Small molecular weight and moderate lipophilicity of MPA make it a good candidate for topical delivery due to good permeability.

**Table 11. Physiochemical characteristics of MPA compared with TAC, and RAPA**

| Drugs | Molecular weight, g/mole | Partition coefficient (log $P_{ow}$), Source: Drug Bank |
|---|---|---|
| Mycophenolic Acid | 320 | 3.8 |
| Tacrolimus | 804 | 3.9 |
| Rapamycin | 914 | 4.85 |

### 3.4.3 In-vitro release study for mycophenolic acid from topical semisolid formulations:

In vitro release studies were conducted to determine the rate of release and permeation of MPA from different pharmaceutical products. The amount of MPA permeated from four different formulations (MPA, 5mg) into the receptor chamber over 24 hours is shown in **Figure 20,** and the permeability parameters are presented in **Table 12**. Formulations tested include Aladerm,

Lipoderm, Versa base, and Emollient base. The MPA permeability parameters (diffusion flux and permeation coefficient) of the products tested can be listed as follows: MPA in Aladerm > MPA in Lipoderm > MPA in Emollient > MPA in Versa Base. Of the four semisolid formulations tested, the cumulative release of MPA from Lipoderm, showed a linear single-phase profile (gradual and sustained release over prolonged period of time) with steady state diffusion flux (1.12 ± 0.24 μg/1.77cm$^2$/hr) and high permeability (6.2 ± 1.3 x10$^{-8}$cm/s) across the biomimetic membrane. MPA in Lipoderm resulted in a moderate amount of MPA being permeated through the membranes by the 24-h time point [42 ± 5μg], while MPA in Aladerm resulted in a high total amount permeated (almost a double that of lipoderm) [76.3 ± 31.5μg], and MPA in Emollient and MPA in VersaBase resulted in a low total amount permeated the membranes by the 24-h time point [18.5 ± 7 and 10 ± 3.3μg, respectively].

**Table 12. Mean steady state diffusion flux, permeation coefficient of MPA, and total amount of MPA permeated from four different formulations into the receptor chamber over 24hr (Mean ± SD, n=3).**

| Formulations | Mean steady state diffusion flux ($J_{ss}$), μg/cm$^2$/hr | Permeation coefficient ($k_p$), cm/s | Total amount permeated over 24hr, μg |
|---|---|---|---|
| MPA in Aladerm | 1.25 ± 0.8 | 6.9 ± 4.5 x10$^{-9}$ | 76.3 ± 31.5 |
| MPA in Lipoderm | 1.12 ± 0.24 | 6.2 ± 1.3 x10$^{-8}$ | 42 ± 5 |
| MPA in Emollient | 0.41 ± 0.2 | 2.2 ± 9.3 x10$^{-9}$ | 18.5 ± 7 |
| MPA in Versa Base | 0.14 ± 0.1 | 7.7 ± 4.1 x10$^{-9}$ | 10± 3.3 |

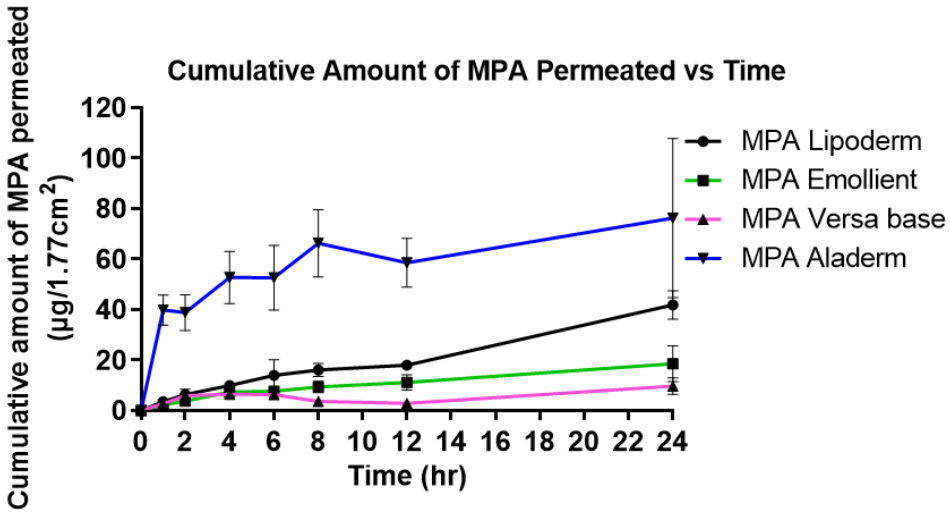



**Figure 20. Cumulative amount of MPA (µg) permeated per 1.77 $cm^2$ from semisolid formulations vs. Time. Data shown as mean±SD, n=3.**

### 3.4.4 Physical characterization of mycophenolic acid in Lipoderm

Mycophenolic acid in Lipoderm demonstrated good homogeneity with absence of aggregates or clumping, and excellent texture with no grittiness. The pH of the formulation was 5.4 ± 0.5 which is comparable to the pH of human skin (4.7) [183, 184].

**Table 13. Physical characteristics of MPA in Lipoderm kept at 25 °C ± 2 °C for a period of 2 and 6 months.**

| Physical characteristics | Immediately after formulation | 2 months | 6 months |
|---|---|---|---|
| Color | W | W | W |
| Liquefaction | – | – | – |
| Phase separation | – | – | – |

− = No change; + = Slight change; W = White

### 3.4.5 MPA in Lipoderm was stable over six months of storage at room temperature

The formulation was kept at room temperature (25 ± 2 °C) for a period of two and six months. Color, liquefaction, and phase separation changes were evaluated, and the data is presented in **Table 13**. Based on the physical assessments performed, MPA in Lipoderm (1%w/w) was shown to be stable for a period of 6 months when kept at the room temperature (25 ± 2 °C). Percent of MPA in Lipoderm immediately after compounding and at 2 and 6 months is shown in **Figure 21.** There was no significant change in the percent of MPA in Lipoderm within the first 2 months and between 2 and 6 months ($p>0.05$). The percent of MPA in Lipoderm remained within the acceptable range (90-110%).

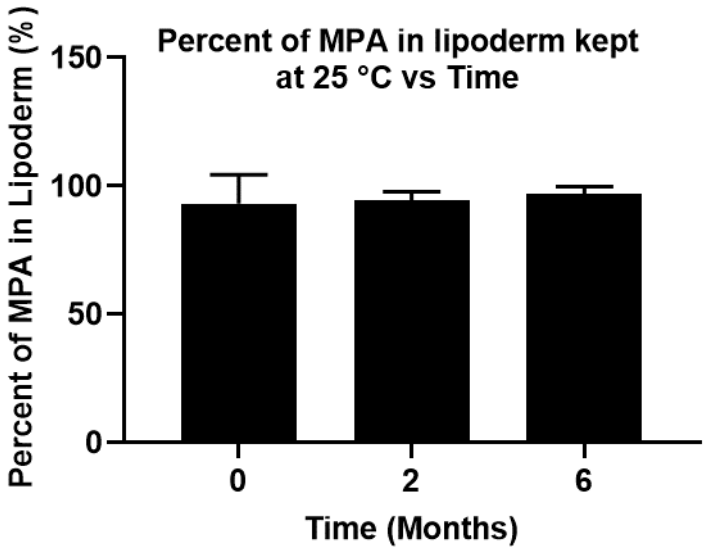



**Figure 21. Percent of MPA in lipoderm kept at 25 °C ± 2 °C immediately after formulation, and at 2 and 6 months. Data shown as mean±SD, n=3. P values were calculated by repeated measures one-way ANOVA with Tukey's multiple comparisons test.**

### 3.4.6 Single topical application of MPA (1 % Lipoderm) results in significantly lower systemic exposure, as compared to the systemic administration

In-vivo pharmacokinetic studies were performed to evaluate the potential for systemic exposure to MPA when the designed product is administered topically. The mean plasma concentration-time profile of MPA after single topical application (1%, 16.6mg/kg), or IV bolus injection (10 mg/kg) is shown in **Figure 22**. Following IV bolus dose, MPA concentrations were high initially (71.8 ± 13 µg/ml) with concentrations declining quickly thereafter over time to reach low values (0.5 ± 0.7 µg/ml) at 24 hours. Following topical dose, the peak MPA concentrations were reached between 3 and 4 hours with 0.6 ± 0.3 µg/ml, with concentrations declining gradually thereafter over time to reach low values (0.2 ± 0.1 µg/ml) at 24 hours. The comparative non-compartmental pharmacokinetic parameters of MPA derived from the plasma concentrations-time data after topical and/or systemic administration of MPA are presented in **Table 14**. Exposure parameters ($C_{max}$, $C_{trough}$, and $AUC_{0\text{-}24hr}$, and F) were markedly lower after topical administration of MPA as compared to systemic administration of MPA (<0.05). This indicates that the systemic drug exposure was minimized by topical administration.

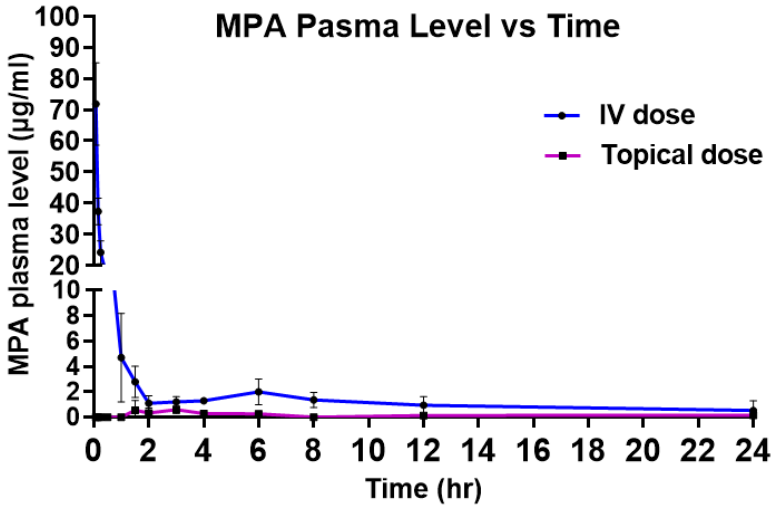



**Figure 22. MPA plasma concentration (µg/ml) –time profiles in Lewis rats following single topical application of MPA (1 % lipoderm, 16.6mg/kg) or IV bolus injection (10mg/kg). Data shown as mean+SD, n=6/Topical MPA group, and n=8/Systemic MPA group.**

**Table 14. Comparative non-compartmental pharmacokinetic exposure parameters (mean + s.d.) following single application of MPA (1 % Lipoderm, 16.6mg/kg) or IV bolus injection of MPA (10mg/kg).**

| PK Parameters | Unit | Systemic administration | Topical administration |
|---|---|---|---|
| $C_{max}$ | µg /ml | 71.8 ± 13 | 0.6 ± 0.2 |
| $T_{max}$ | hr | 0.08 | 3 |
| $C_{trough}$ | µg /ml | 0.6 ± 0.3 | 0.2 ± 0.1 |
| $AUC_{0\text{-}24hr}$ | µg.hr/ml | 33.5 ± 7.7 | 5 ± 3.2 |
| $AUC_{0\text{-}24hr}$/Dose | | 11 ± 2.6 | 1.02 ± 0.6 |
| F | % | 100 | 9 |

### 3.4.7 Single topical application of MPA (1 % Lipoderm) results in significantly higher local tissue concentrations, as compared to systemic administration.

In-vivo pharmacokinetic studies were performed to evaluate the tissue drug concentrations achieved following product exposure, which can directly impact the product efficacy. MPA concentrations in skin, muscle, and DLNs (that target tissues for pharmacological effect) at 24 hours after single application of MPA (1 % Lipoderm, 16.6mg/kg) or IV bolus (10mg/kg) is presented in **Figure 23**. MPA concentrations in skin, muscle, and DLNs after single application of MPA Lipoderm were significantly higher than the values obtained after systemic administration (<0.05), which indicates the local drug delivery to the site of application after topical drug administration. Tissue concentrations at the application site were in this order, from highest to lowest: the skin, muscle, and DLNs, which indicates that TAC penetrated the muscle and DLNs to a lesser degree than the skin, and most of the drug is retained in the skin layers. Systemic administration of MPA is unlikely to achieve high tissue concentrations particularly in the skin due to the extensive systemic distribution and elimination. The tissue to plasma ratios at 24 hours following single application of MPA (1% Lipoderm, 16.6mg/kg) or IV bolus injection (10mg/kg) are presented in **Table 15.** The tissue to plasma concentration ratios after topical application are significantly higher than the tissue to plasma concentration ratios after systemic administration. This indicates the permeation and accumulation of MPA into the local tissues particularly skin after topical drug administration with minimal systemic exposure.

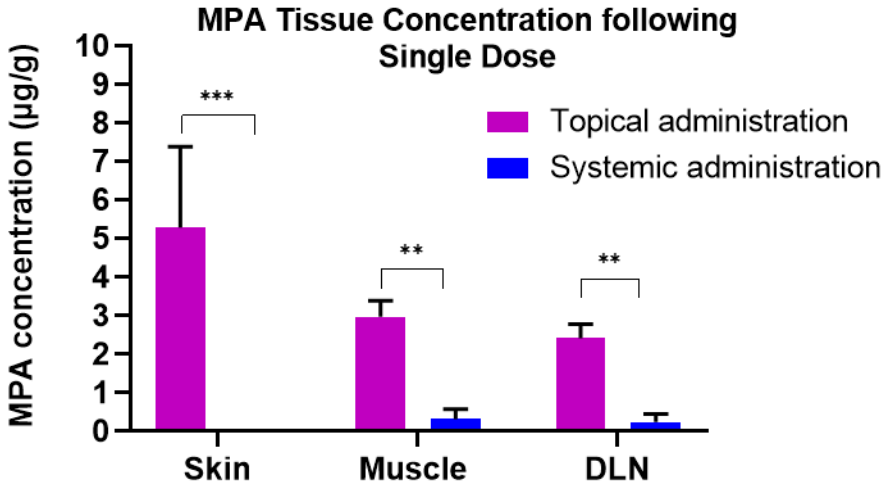



**Figure 23. MPA concentrations (µg/g) in skin, muscle, and draining lymph nodes at 24 hours after single topical application of MPA (1% Lipoderm, 16.6mg/kg) or IV bolus (10mg/kg) in rats. Asterisks indicate statistical significance. **p<0.01, ***p<0.01 significantly different from IV administration. Data presented as mean ± SD, n=3. P values were calculated by student t test.**

**Table 15. MPA Tissues to plasma distribution ratios at 24 hours following single topical application of MPA (1% Lipoderm, 16.6mg/kg) or IV bolus of MPA (10mg/kg).**

| Tissue -plasma concentration ratios | Systemic Administration | Topical Administration (Application Site) |
|---|---|---|
| Skin-plasma concentration ratio | 0 | 52 |
| Muscle-plasma concentration ratio | 1.5 | 29 |
| DLNs-plasma concentration ratio | 1.1 | 24 |

### 3.4.8 Single topical application of MPA (1% Lipoderm) results in significantly higher local tissue concentrations, as compared to the concentrations at the contralateral sites.

MPA concentrations in skin, muscle, and DLNs collected from the application limbs and contralateral limbs following a single application of MPA (1% Lipoderm, 16.6mg/kg) are presented in **Figure 24**. Mycophenolic acid concentrations in skin, muscle, and DLNs collected from the application limb are significantly higher than MPA concentrations in skin, muscle, and DLNs collected from the contralateral limb ($p<0.5$). This indicates that MPA mainly localizes to the site of topical application with limited distribution to other sites remote from the site of application.

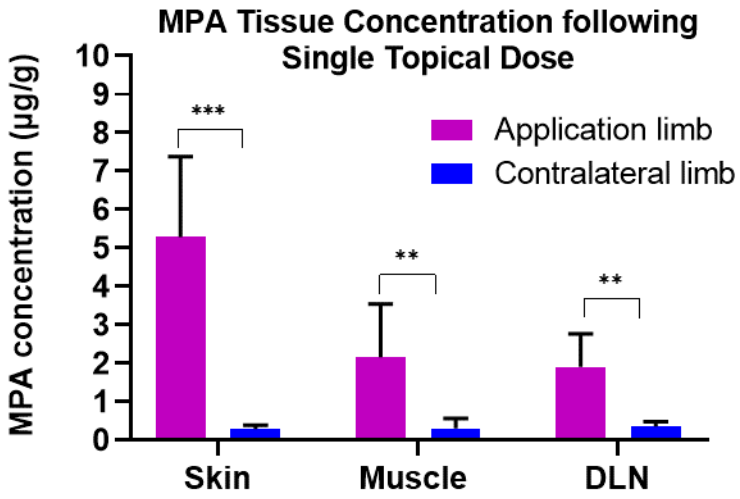



**Figure 24. MPA concentrations (µg/g) in skin, muscle, and draining lymph nodes collected from the application limb and contralateral limb at 24 hours following single application of MPA (1% lipoderm, 16.6mg/kg) in rats. Asterisks indicate statistical significance. **p<0.01, ***p<0.01 significantly different from the contralateral leg. Data presented as mean ± SD, n=3. P values were calculated by paired t test.**

### 3.4.9 Time course of MPA concentrations in skin and muscle following single topical application of MPA (1% Lipoderm).

In order to assess MPA's distribution in the local tissues after topical administration, time course of MPA concentrations in skin and muscle at 2, 6, 12, and 24 hours following single application of MPA (1% Lipoderm, 16.6mg/kg) is evaluated and presented in **Figure 25**. Pharmacokinetic parameters of MPA in the skin and muscle following a single application of MPA (1% Lipoderm, 16.6mg/kg) are presented in **Table 16.** Peak concentrations of MPA in skin and muscle were reached 2 hours post topical dose administration (17±4.4 and 6.6±1.6 µg/g respectively), then concentrations gradually declined over time to reach low concentrations at 24 hours post-topical dose administration (4.6 ± 1 and 2.2 ± 1 µg/g respectively). Peak concentrations of MPA in plasma were reached 3 hours post topical dose administration (0.6 ± 0.2 µg/ml), then concentrations gradually declined over time to reach low concentrations at 24 hours post-topical dose administration (0.2 ± 0.1 µg/ml). MPA exposure as measured by $AUC_{0\text{-}24hr}$ in the skin and muscle was significantly higher than the values observed in the plasma ($p<0.05$). MPA exposure ($AUC_{0\text{-}24hr}$) in the skin were significantly higher than the values observed in the muscle ($p<0.05$).

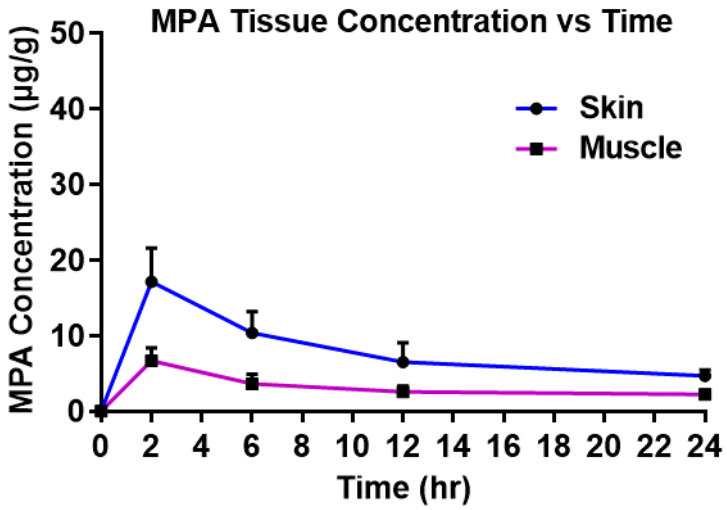



**Figure 25. Time course of MPA concentrations (µg/g) in skin and muscle following a single topical application of MPA Lipoderm (1%, 16.6mg/kg) in rats. Data presented as mean ± SD, n=3.**

**Table 16. Comparative non-compartmental pharmacokinetic exposure parameters of MPA (mean + s.d.) in the skin, muscle, and plasma and tissue to plasma concentration ratios following single topical dose (MPA Lipoderm 1%, 16.6mg/kg).**

| | $AUC_{0\text{-}24hr}$ (µg.hr/ml) | $C_{max}$ (µg/g or µg/ml) | $C_{trough,\ 24h}$ (µg/g or µg/ml) |
|---|---|---|---|
| Skin | 189 ± 46 | 18 ± 4.4 | 5 ± 1 |
| Muscle | 74 ± 19 | 6.6 ± 1.6 | 2.2 ± 1 |
| Plasma | 5 ± 3.2 | 0.6 ± 0.2 | 0.2 ± 0.1 |
| Skin-blood ratio | 58 | 29 | 64 |
| Muscle-blood ratio | 26 | 11 | 22 |

### 3.4.10 Average daily trough MPA concentrations in plasma after topical application (1% Lipoderm) was significantly lower than the values observed after systemic administration

In this study, MPA Lipoderm (1%) was administrated daily for 7 days. As shown in **Figure 26**, Trough plasma concentrations of MPA after daily topical applications of MPA (1% Lipoderm, 16.6mg/kg) for 7 days ranged between 0.05 and 0.124µg/ml, while trough plasma concentrations of MPA after daily intraperitoneal injections (10mg/kg) for 7 days ranged between 0.26 and 0.36µg/ml. Topically treated animals had significantly lower trough plasma concentrations compared with systemically treated animals (P<0.0001).

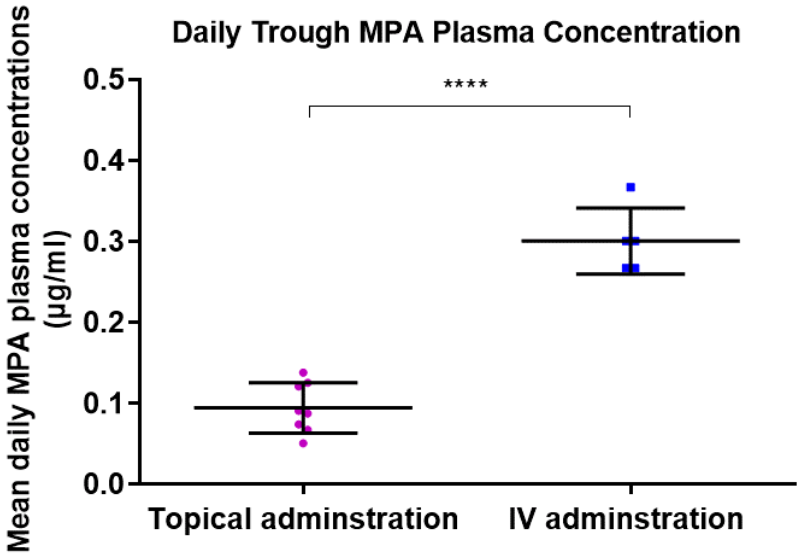



**Figure 26. Average daily trough concentrations of MPA (µg/ml) following daily topical application of MPA (1% lipoderm, 16.6mg/kg) or daily intraperitoneal dose (10mg/kg) for 7 days (trough levels) are compared. Each data point represents the mean value of MPA measurements acquired every day until day 7. Statistical analyses of the differences between the 2 groups are shown. Data presented as mean ± SD, n=3/group. are indicated, ****P<0.0001 by student t test.**

### 3.4.11 Systemic exposure to MPA following single topical application or repeated topical application of MPA (1% Lipoderm) for 7 days

Systemic exposure as measured by mean area under the curve ($AUC_{0–24hr}$) of MPA following single topical application (Day 1) or daily topical application of MPA (1% Lipoderm, 16.6mg/kg) for 7 days (Day 7) is shown in **Figure 27**. Despite that the mean $AUC_{0-24h}$ increased from 5 ± 3.2 µg·h/ml on day 1 to 16.5 ± 7 µg·h/ml on day 7, the difference was not statistically significant ($p>0.05$) due to the sample size and variability. The estimated ratio of accumulation from day 1 to day 7 was 2.6. This indicates that there was systemic accumulation of MPA following repeated topical application of MPA at this dose. However, these values are significantly lower than the values observed after the first systemic dose (33.5 ± 7.7 µg.h/ml).

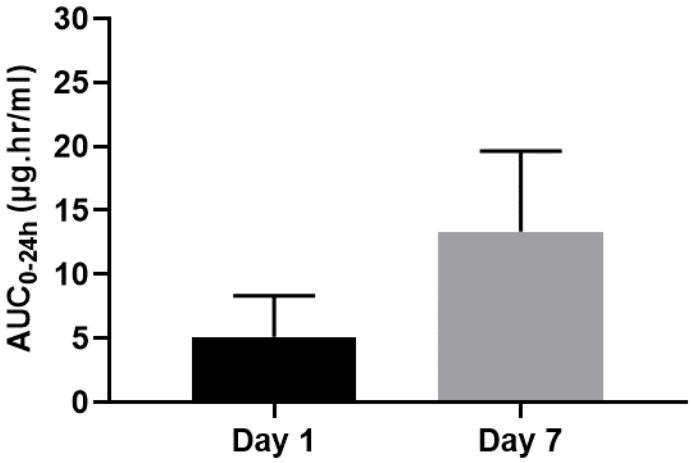



**Figure 27. Mean area under the curve ($AUC_{0–24hr}$) of MPA following single topical application (Day 1) or daily topical application of MPA (1% Lipoderm, 16.6mg/kg) for 7 days (Day 7). Statistical analyses of the differences between the 2 groups are shown. Data presented as mean ± SD, n=3. are indicated, P>0.05 by Mann Whitney test.**

## 3.5 Discussion

Upon topical application, MMF undergoes only limited or unpredictable metabolism to the active form MPA in the skin. This is because, skin esterase activity levels are variable [184]. Our study takes the logical approach to create/develop topical formulations with MPA rather than MMF, to circumvent the confounder of skin esterase activity. Topical application of the active form, MPA via the skin or mucosa in VCA could improve effectiveness by mainly concentrating the drug in the graft, decreasing systemic exposure, and the off-target effects. The use of topical MPA in VCA could have additive benefits when combined with topical TAC or other immunosuppressants. It could help minimize the need for systemic MMF, TAC, and corticosteroids for the prevention/treatment of allograft rejection, and augment efficacy and medication adherence in patients, while lowering risk of systemic adverse effects.

Topical delivery of MMF has been attempted in dermatology for patients with psoriasis, vitiligo, atopic dermatitis, or allergic contact dermatitis with varying results [185-188]. Although MMF is relatively lipophilic (LogP 2.5) with moderate molecular weight (437.525 g/mol), skin permeation may be challenging due to the stratum corneum (SC) which is a natural barrier that limits drug absorption and exposure. A thickened SC is the cause of treatment failures in psoriasis with topical MMF [189] requiring the need for penetration enhancers such as eucalyptol (EUL) and N-methyl-2-pyrrolidone (NMP) [190]. However, these enhancers themselves can cause skin irritation [191]. To date, no study has compared different formulation bases for topical delivery of MMF or MPA either in dermatology or in VCA applications. Also, to our knowledge, a formal analysis of the pharmacokinetics and bioavailability of the active form of MPA especially across the skin barrier has not been previously reported.

Ideally, topical delivery should be tested across a skin barrier. Excised human skin is considered the gold standard model for in-vitro drug permeation and penetration assessment. However, large variations are common across human skin explants due to differences in age, gender, race and donor anatomical site. On the other hand, animal skins from pigs (porcine ear), guinea pigs, hairless mice or snakes (ecdysial skin) have been used as predictive model systems for in vivo human permeation/ penetration of topical agents [192, 193]. But, there is significant intra- and inter-individual variation between animal and human skin, when skin characteristics, such as the thickness of skin (especially SC), lipid content, density of hair follicles, and esterase enzyme activity in each model are compared [194]. Studies have shown that the skin of rodents, such as hairless rats and hairless mice, are more permeable than human skin using drugs/agents with different physicochemical properties [195]. Some of the critical parameters that cause such variability in permeation/penetration profiles in animal or human skin are effects of storage and freezing (use of cryopreserving agents such as 10% glycerol) that can cause alterations in skin hydration or electrical resistance [195-197]. This can alter permeability and the lag time of hydrophobic drugs such as MPA as tested in this study. Finally, there is no consensus on the use of an ideal cryoprotectant for skin preservation or the optimal storage time/conditions for frozen skin used for in-vitro drug permeation/penetration studies. To overcome these individual limitations with animal or human skin, and to optimize testing of the topical MPA delivery parameters, our study combined in-vitro and in-vivo evaluation of safety and feasibility of topical MPA for skin applications.

We first evaluated our formulations in-vitro in a FDCS system across a regenerated cellulose dialysis membrane (SpectraPor® RC) that functions like a biomimetic skin barrier. Our choice of the FDCS system was based on its validated metrics (such as membrane parameters, cell

dimensions, temperature, membrane treatment, stirring efficiency, sampling frequency). Using biomimetic membrane shown to be suitable for this study in terms of reproducibility, consistency, and data variation [198]. Saturated MPA concentration (10mg/ml) was used to ensure drug delivery across the membranes. MPA in solution exhibited a good steady state diffusion flux (3.8 ± 0.1 µg/1.77cm$^2$/hr) and permeation ($1.1x10^{-7}$± $3.2x10^{-9}$ cm/s) across the biomimetic membrane in a linear fashion (Fick's law of diffusion). Saturation or plateau state was not reached in 24 hours.

Careful correlation of characteristics such as permeability coefficient, diffusivity, and partition coefficient for each of the four semisolid formulations tested were important considerations in our study. Release of MPA from the four formulations seemed to be affected by the formulation properties. The highest initial release rate, mean steady state flux, permeation, and total amount permeated over 24 hours were seen with MPA in Aladerm, followed by MPA in Lipoderm, and then MPA in emollient and MPA in VersaBase. MPA in Aladerm exhibited the highest diffusion and fast initial release (burst) and total amount permeated to the receptor (almost a double that of MPA in Lipoderm). This could indicate a propensity for rapid systemic absorption and high exposure with clinical use. Furthermore, Aladerm has a fluid texture that may lead to difficulty in application and maintaining the formulation on the skin for reliable absorption and efficacy. We excluded MPA in Aladerm from further testing. Similarly, we excluded MPA in emollient and Versa Base as choices for further development because of their thick and greasy nature, which challenges skin penetration and topical application (difficulty in washing, staining of clothes and reduced patient adherence) [199, 200].

A moderate amount of MPA permeated into the receptor chamber over 24hr with the Lipoderm formulation. The timeline and kinetics of drug diffusion and release were optimal with MPA in Lipoderm (1w/w). This included gradual diffusion and sustained drug release which

facilitates a prolonged local site-specific action of the drug. Also, the formulation was stable (with no degradation or alterations in pH or composition) over 6 months of storage at 25 °C. The pH of the formulation was close to natural skin pH (4.7) [183] minimizing risk of skin irritation. Additionally, the prepared formulation (1% w/w) showed good physical characteristics and stability over 6 months of storage at 25 °C. No change in color, liquefaction, or phase separation was observed. Percent of MPA in Lipoderm remained within the acceptable range (90-110%). This indicates that the prepared formulation could maintain a good quality and efficacy. While a high percentage of MPA dose was released from MPA in solution, small percentage was permeated into the receptor chamber and the rest was remained in the biomimetic membrane. However, in comparison to MPA in solution, we observed slower flux and lower permeability of MPA from all the semisolid formulations across the biomimetic membrane. Every formulation of topical MPA has different properties and may permeate the membrane at different rates depending on its components.

In-vivo pharmacokinetic study was performed to evaluate the dermal absorption of MPA and the potential for systemic exposure to MPA when the prepared formulation is administered topically. Additionally, the study evaluated the tissue drug concentrations achieved following product exposure, which can directly impact the formulation efficacy. The results demonstrated that systemic exposure as measured by $AUC_{0\text{-}t}$, $C_{max}$, and $C_{trough}$ after single topical application of MPA were significantly lower than the values observed after systemic delivery of MPA (Figure 22; Table 14). This indicates that the systemic drug exposure was reduced by topical administration. Low concentrations of MPA were observed in the tissues (skin, muscle, DLNs) after 24 hours with a single IV dose. Systemic administration of MPA may not provide high concentrations in the tissues due to the rapid disposition of the drug from the blood. Conversely,

the low systemic bioavailability of MPA that observed after topical administration (9%) was due to the drug accumulation in the local tissues at the site of application. Average daily MPA concentrations in plasma showed that topically treated animals had significantly lower plasma concentrations of MPA when compared to the systemically treated animals (3.7-fold lower). MPA concentrations in the local tissues (skin and muscle) collected from the application site after topical administration were significantly higher than values observed after systemic administration (Figure 23). The SC barrier of the skin may also slow or limit the rate of systemic drug absorption and release into the systemic circulation. In fact, the skin component retained most drug with topical application of MPA in Lipoderm when concentrations across skin, muscle, DLNs and plasma were compared (Table 15).

MPA concentrations in skin and muscle reached the highest values within 1-2 hours post-topical dose administration, which indicates the rapid absorption of the drug into the skin and muscle tissue. MPA concentrations gradually declined to reach low concentrations at 24 hours post-topical dose administration due to drug clearance into the blood. Results showed that topical application of MPA (Lipoderm 1%) at a dose of 16.6mg/kg provides high local tissue drug exposure at the site of application and low systemic exposure. Most of the amount applied was absorbed within 24 hours. Systemic exposure to MPA (1% Lipoderm, 16.6mg/kg) after repeated topical applications was higher (2-fold higher) when compared to the values observed after single topical application (Figure 27) indicating moderate systemic accumulation of MPA after repeated topical applications. However, the dosing regimen can be further modified to minimize the drug accumulation after repeated applications.

MPA concentration in local tissues (skin, muscle, and DLNs) collected from the application site was significantly higher than MPA concentrations in tissues collected from the

contralateral site (18, 7, and 5-fold higher) (Figure 24). This confirms that MPA mainly localizes to the site of topical application with limited diffusion to the blood and the other contralateral sites remote from the site of application.

The high MPA concentration in DLNs may relate to the hydrophobic/lipophilic nature of MPA. It is known that lipophilic agents are preferentially taken up by the lymphatic system and the degree of uptake depends on factors such as particle size (size range 200-600 nm), surface charge, molecular weight, and hydrophobicity [201, 202]. DLNs are the initial site of allorecognition and T cells activation, and thereby localization of higher concentrations of MPA in these tissues could curb innate or adaptive immune responses in VCA tissues.

The results of this study show that topical application of MPA (Lipoderm 1%) in a dose of 16.6mg/kg/day is effective in achieving high local tissue concentrations with low plasma levels. Despite that the lowest trough MPA plasma levels at which systemic immunosuppressive effects can be observed is not known, studies have reported that doses between 10-20mg/kg (systemic MPA) are well-tolerated doses that are used in rat models of solid organ allotransplantation [203]. Our results showed that systemic administration of MPA at dose of 10mg/kg/day provides trough MPA plasma levels ranged between 0.3-0.4µg/ml. Our results show that the average MPA concentrations in skin, muscle, and DLNs at 24 hours after single topical application of MPA (1% Lipoderm, 16.6mg/kg) were significantly higher than the values observed following daily systemic administration of MPA at dose of 10mg/kg/day. Further studies should be performed to determine the target effective therapeutic tissue concentrations and to evaluate the efficacy of MPA (1% Lipoderm, 16.6mg/kg) in preventing skin rejection in a rat model of VCA.

A topical formulation of MPA in Lipoderm (1%w/w) with good in vitro/in vivo characteristics and without local adverse effects was developed. The formulation was stable over

6 months of storage at 25 °C. MPA in Lipoderm (1%w/w) is a well-tolerated formulation for local delivery of MPA. This confirms the feasibility of topical application of MPA for site-specific immunosuppression and enables future wider applications. MPA in Lipoderm can be combined with other topical immunosuppressants such as TAC for synergistic efficacy on T cell responses. This can result in more rapid onset, increased efficacy, reduction of systemic immunosuppression levels, and thus improving the patient compliance.

# 4.0 Combined Treatment of Topical Tacrolimus and Mycophenolic Acid with Low-Dose of Systemic Tacrolimus Prolongs Survival of Vascularized Composite Allografts without Systemic Adverse Effects

## 4.1 Abstract

Use of topical immunosuppressants has been reported in experimental and clinical VCA, and the results support the concept of site-specific immunosuppression, and suggest potential benefits in VCA. This study was performed to evaluate whether combined treatment of topical TAC and MPA applied at the transplant site in conjunction with low dose systemic immunosuppression with TAC can be effective in sustaining VCA graft survival and in reducing systemic morbidity. Orthotopic hind limb allotransplants (Brown Norway (BN) to Lewis (Lew) rats) were performed. Group 1 was treated with no drugs (Control). Other groups were treated with systemic TAC (STAC) at dose of 1mg/kg/day for 7 days. On post-operative day (POD) 8, STAC dose was dropped to 0.1mg/kg for Group 2 and maintained at 1 mg/kg for Group 3. Topical application of TAC and MPA on transplanted (Tx) limb (Group 4) was initiated on day 8 without systemic immunosuppression with TAC. Topical application of TAC and MPA on non-Tx limb (Group 5) or on Tx limb (Group 6) were initiated on day 8 to overlap with low dose STAC (0.1mg/kg/day). Treatment was continued until grade 3 rejection or > 100 days survival. Blood/plasma and allograft tissue levels of TAC and MPA were measured using LC-MS/MS. Systemic toxicity markers were evaluated. The immunomodulatory effect of TAC and MPA was assessed by flow cytometry, and skin grafting. Animals treated with low dose of STAC, topical therapy on Tx limbs, or low dose of STAC with topical therapy on nonTx limb rejected their allografts by 37, 24, and 60 days. Optimal outcomes were achieved in the animals treated with topical TAC and MPA applied at the transplant site in conjunction with low dose of STAC with allograft survival >100 days without systemic side effects (nephrotoxicity or diabetogenicity). Concentrations of TAC and MPA were significantly higher in the allograft, particularly in skin when compared to the concentrations observed in the blood (TAC: 250-fold higher; MPA: 480-

fold higher). We conclude that combined treatment of topical TAC and MPA applied at the transplant site with low dose of STAC can be an effective therapeutic approach to sustain VCA graft survival to reduce systemic morbidity. The allograft survival was not related to the induction of donor-specific tolerance but to the long-lasting high loco-regional concentrations of TAC and MPA in the allograft, particularly the skin. There was survival benefit of applying the topical therapy in the allograft as compared to a remote site. These observations establish the basis for further investigation in clinical VCA.

## 4.2 Introduction

TAC and MMF/MPA have been widely used as effective dual maintenance systemic immunotherapy in SOT and VCA. This is due to a synergistic effect based on different mechanisms of action [204]. Although this immunosuppressive protocol has been successful in preventing allograft loss, it has not completely prevented AR of the skin [64, 205]. Additionally, systemic use of TAC and MPA is associated with several morbidities including infections, malignancy, and organ damage. Topical immunotherapy is a potential therapeutic option to provide site specific immunosuppression, with minimal systemic exposure and toxicity [104]. Topical therapy combining TAC with MPA could have synergistic benefits by targeting different mechanistic pathways and molecular targets in the skin [206]. This can result in rapid onset of action, increased efficacy, and reduction of systemic immunosuppressive drugs.

Topical application of immunosuppressive drugs has been reported in experimental VCA. Topical therapy fails to prevent allograft rejection when used alone. In an earlier work, our group reported that daily topical TAC is capable of locally inhibiting the immune cells, delaying skin rejection, and improving allograft survival after a short course of systemic CsA [104]. Optimal outcomes have been achieved when topical therapy is applied following or in combination with systemic therapy. Improved face allograft survival has been achieved with systemic CsA. A followed by topical tacrolimus or clobetasol as compared to systemic CsA therapy alone [207]. Topical steroids have been also used to prolong the survival of skin [208] and hind limb allografts [135] when preceded by systemic therapy but with local side effects. Topical CsA was capable of locally inhibiting the immune cells after a short course of low systemic CsA. Levels of CsA were high in skin and low in blood [209]. Topical therapy has been routinely used for treating dermatologic diseases [210, 211]. It has been also used in clinical VCA, but mainly as needed (if

AR episodes occurs) with unclear or unexplored beneficial effects [11]. It has been reported that grade 1-2 rejections could be treated with TAC ointment twice a day (Protopic®, 0.1%) and clobetasol 0.05% cream twice a day (Temovate®) without increased doses of systemic drugs. Others have reported that IV steroids and increased doses of systemic TAC are required to reverse rejection episodes in addition to topical TAC [212].

However, synergistic benefits of therapeutic combination of topical immunosuppressants (TAC and MPA) with low dose of systemic immunosuppression with TAC have not been studied in VCA. TAC and MPA have different mode of action and may act synergistically. A treatment combining MPA and TAC exhibited a significant inhibitory effect on allo-reactive T cell responses while maintaining regulatory T cells [213]. Therefore, renal transplant patients treated with MPA in combination with TAC required lower doses of TAC to maintain the allografts as compared to patients treated with only TAC [213].

The aim of this investigation was to document this presumed synergistic effect. We evaluated whether combined treatment of topical TAC and MPA applied at the transplant site in conjunction with low dose systemic immunosuppression with TAC can be effective in sustaining VCA graft survival and in reducing systemic morbidity in a low-dose combination corticosteroid-free regimen in a clinically relevant model of VCA.

## 4.3 Materials and Methods

### 4.3.1 Animals

All animal experiments were performed under a protocol approved by the institutional animal care and use committee (IACUC) at the University of Pittsburgh. Male Lewis rats (recipients) and male Brown Norway rats (donors) aged 8 to 10 weeks, weighing about 250 to 300 g, were purchased from Charles River Laboratories (Horsham, PA) and housed in a specific pathogen-free barrier facility and maintained in accordance with IACUC guidelines. This combination represents a full major histocompatibility complex mismatch. Animals were housed individually, and plastic Elizabethan collars were used to prevent oral ingestion of the topical formulations, and to prevent access of animal to the application site. All procedures were in a compliance with American Association for the Accreditation of Laboratory Animal Care (AALAC) recommendations and the principles set forth in the National Institute of Health Publication 80-23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended.

### 4.3.2 Orthotopic Hind-Limb Transplantation from Brown Norway to Lewis Rats

Hind limbs from donor Brown Norway (BN) rats were transplanted to recipient Lewis (Lew) rats as shown in **Figure 28**. In brief, donor and recipient animals were anesthetized with Nembutal (50mg/kg) or Ketamine (80mg/kg). Donor operations: The skin was incised proximal to the mid-thigh area. After exposing and cutting the femoral artery, vein, and nerve, the individual muscle groups and the femur were cut at the mid-shaft. The limb was flushed with heparinized

Ringer's Lactate until clear fluid came from the vein. Recipient operation: After removing the similar portion of the leg, the donor leg was attached. Femoral bone osteosynthesis was achieved using an 18-gauge needle as an intramedullary rod. Femoral vessels were anastomosed using 10-0 Nylon interrupted sutures. The muscles were approximated using 5-0 vicryl and the skin were closed using 5-0 polyamide monofilament interrupted sutures [214].

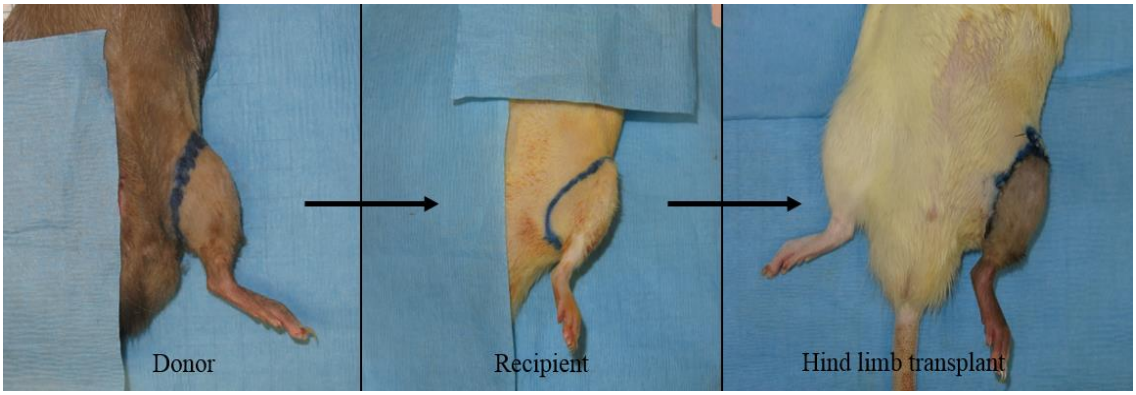



**Figure 28. Brown Norway to Lewis hind limb transplant.**

### 4.3.3 Study Design (Groups and immunosuppression regimen)

To test the hypothesis that combined topical therapy (TAC and MPA) applied at the transplant site in conjunction with low dose of systemic immunosuppression with TAC sustains the survival of VCA graft in a clinically relevant VCA model, Lew rats (recipients) underwent orthotopic hind-limb allotransplantation as previously described earlier. Transplanted animals in Group 1 (control) didn't receive any treatment. Other transplanted animals were treated with TAC interperitoneally at a dose of 1mg/kg/day for 7 days. On day 8, transplanted animals in different group were treated with different immunosuppressive protocols. Treatment was continued to the

study's end point (Grade 3 rejection or > 100 days survival). Groups and treatments are described in **Table 17.**

**Table 17. Groups and treatments. All transplanted animals (except animals in Group 1) were treated with TAC, interperitoneally in a dose of 1mg/kg/day for 7 days post-transplant. On day 8, transplanted animals belong to each group were treated with different immunosuppressive regimen.**

| Groups | # of animals | Immunosuppression regimen |
|---|---|---|
| Group 1 | 5 | No treatment |
| Group 2 | 6 | TAC (0.1mg/kg/day), interperitoneally to the end point |
| Group 3 | 6 | TAC (1mg/kg/day), interperitoneally to the end point |
| Group 4 | 4 | Topical TAC + Topical MPA applied on Tx limb (no systemic therapy) |
| Group 5 | 5 | TAC (0.1mg/kg) interperitoneally combined with Topical TAC + Topical MPA applied on non-Tx limb |
| Group 6 | 6 | TAC (0.1mg/kg) interperitoneally combined with Topical TAC + Topical MPA applied on the Tx limb |

### 4.3.4 Drugs and Drug Administration

Tacrolimus was prepared in vehicle consisting of 0.8% ethanol, 0.2% Cremophor EL (kolliphore®), and saline solution (Sodium chloride, 0.9% w/v, USP) for intraperitoneal administration in a final concentration of 1 mg of tacrolimus/ml. The tacrolimus solution was administered at a volume of 1 ml/kg for a dose of 1 mg/kg in groups (2, 3, 4, 5, and 6), and at a volume of 0.1ml/kg for a dose of 0.1 mg/kg in groups (2, 3, 5, and 6). 0.5g tacrolimus ointment (Protopic®, 0.03%) was applied daily at an effective dose of 0.5 mg/kg in groups 3, 4, 5, and 6.

0.5g Mycophenolic acid in lipoderm (1%) was applied daily at an effective dose of 16.6 mg/kg in groups 4, 5, and 6. TAC and MPA ointments were applied evenly on the allografts with massaging. TAC and MPA formulations were applied at different times in order to minimize the risk of transdermal drug interaction (e.g. alter release kinetics) and systemic absorption due to concurrent application of TAC and MPA formulations, Tacrolimus ointment was applied 12 hours after MPA in Lipoderm. Mycophenolic acid and tacrolimus powder, cermophore oil (kolliphore®), and propylene glycol USP were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tacrolimus ointment (Protopic®, 0.03%) was commercially prepared by Astellas Pharma US in a base of mineral oil, paraffin, propylene carbonate, white petrolatum, and white wax. Mycophenolic acid in lipoderm (1%) was compounded by Hieber's Pharmacy, Pittsburgh, PA. Lipoderm was manufactured by PCCA (Professional Compounding Centers of America). Drugs and immunosuppression regimen are described in **Table 18**.

**Table 18. Drugs and Drug Administration**

| **Drugs** | **Dose** | **Drug administration** | **Duration** |
|---|---|---|---|
| Tacrolimus | 1mg/kg/day | Intraperitoneal | Treatment continued until Grade 3 rejection or > 100 days allograft survival. |
| | 0.1mg/kg/day | Intraperitoneal | |
| Tacrolimus (Protopic®) | 0.5mg/kg/day, ointment 0.03% | Topical application | |
| Mycophenolic acid | 16.6mg/kg/day, lipoderm 1% | Topical application | |

### 4.3.5 Graft Survival Evaluation

Animals were monitored daily for clinical signs of rejection. Grade 0. No sign of rejection. Grade 1. Erythema. Grade 2. Progressive erythema and edema. Grade 3. Skin slough/epidermolysis. Grade 4. Mummification and necrosis as described previously [60, 214] and shown in **Figure 29**. Grade 3 rejection or > 100 days survival was defined as the end point of the study.


**Figure 29. The clinical stages of limb rejection. Animals receiving a hind limb VCA were monitored and scored on a four-point rejection scale.**

### 4.3.6 Histopathology Evaluation

Skin and muscle biopsies were taken from the allograft at the end point using a five-mm punch, fixed in 10% neutral buffed formalin, embedded in paraffin, and sectioned. Three-µm sections mounted on separate slides and were stained with hematoxylin and eosin (H&E) for microscopic examination. Histopathological analysis was performed by a transplant pathologist who is blinded to the groups and treatments, and rejection was assessed according to the BANFF classification for rejection as presented in **Table 19 and 20** [60, 214].

**Table 19. Histologic classification of skin rejection**

| Grade of skin rejection | Histological characteristics |
|---|---|
| Grade 0 | No lymphocytic infiltrate |
| Grade 1 | Perivascular lymphocytic and eosinophilic infiltrates |
| Grade 2 | Additional interphase reaction in epidermis and/or adnexal structures |
| Grade 3 | Diffuse lymphocytic infiltration of epidermis and dermis |
| Grade 4 | Necrosis and loss of the epidermis |

**Table 20. Histologic classification of muscle rejection**

| Grade of muscle rejection | Histological characteristics |
|---|---|
| Grade 0 | No lymphocytic infiltrate |
| Grade 1 | Mild perivascular lymphocytic infiltrates, mild edema |
| Grade 2 | Edema, myocyte necrosis, lymphoid infiltrate |
| Grade 3 | Necrosis, vascular thrombosis, muscle replacement by fibrous tissues |

### 4.3.7 Flow Cytometric Analysis

Flow cytometry was performed to quantify levels of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs) in the peripheral blood at post-operative days 30 and 120 for group 2, 3 and 6. Briefly, peripheral blood (300µl) was collected from the tail vein. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient density centrifugation method. For T-reg analysis, PBMCs were stained with fluorochrome-conjugated mouse anti rat monoclonal antibodies for cell surface markers (CD11b/c, CD3, CD4, CD25, and CD45) or intracellular markers (FoxP3). These antibodies were added at the optimized concentrations stored on ice for 30 minutes, washed twice,

and analyzed by flow cytometry using LSRII flow cytometer (BD Biosciences, San Jose, CA) using an open gate excluding small debris. PBMCs from naïve Lewis rats were used as negative control. Stained cells were run on LSRII-flow cytometer (BD Bioscience, San Diego, CA) and data were analyzed using FlowJO (Tree Star, Ashland, OR). Antibodies in various fluorochrome combinations were purchased from BD Biosciences (San Diego, CA).

### 4.3.8 Full-Thickness Skin Grafting for Recipient Tolerance Challenge

Secondary skin graft challenge was performed to evaluate donor-specific in vivo tolerance in animals (n=3) with long-term surviving allografts at post-operative day 135. Skin allografts with a dimension of 2x2cm were harvested from the back of the same strain (Lew), donor strain (BN) or third-party strain rats (Wister Furth, WF) and transplanted on to the back of the animals with long-term survival allografts using skin sutures (5-0). Grafts were bolstered in place for 5 days, and subsequently evaluated daily for signs of rejection. Rejection was defined as hair loss, epidermolysis and desquamation of the skin graft.

### 4.3.9 Signs of Nephrotoxicity Evaluation

Kidney function was evaluated by measuring creatinine clearance (CrCL) on the day before surgery (day -1), and on day 120 after surgery. Twenty four-hour urine collections were performed to measure urinary creatinine levels (urine Cr), and blood samples were collected for serum creatinine levels (serum Cr). Creatinine levels were analyzed by standard clinical chemistry methods in the Central Laboratory of University of Pittsburgh Medical Center (UPMC). CrCl,

(ml/min) was calculated (Urine Cr x Urine volume)/Serum Cr)/1440) [215, 216]. Results were compared to naïve age-matched Lewis rats.

### 4.3.10 Signs of Hyperglycemia Evaluation

Intraperitoneal glucose tolerance test (IPGTT) was performed on day 125 for animals in groups 3 and 6. Animals were fasted for 12 hours, and glucose (2g/kg of body weight) was administered. Blood glucose concentration was measured in the venous blood collected from the tail vein using Accu-chek® sensor at 0, 30, 60, and 120 min after the intraperitoneal injection of glucose. Established standard criteria, diabetes: fasting glucose >126 mg/dL or glycemia at 120 min (IPGTT) >200 mg/dL; pre-diabetes: fasting glucose >100 and < 126 mg/dL or glycemia at 120 min (IPGTT) >140 and <200 mg/dl [217, 218]. We also monitored the animal feeding behavior and the percent change in the body weight.

### 4.3.11 Quantification of TAC and MPA Concentration in Tissues

The skin sites for tissue sampling were wiped down three times with ethanol-soaked gauze to remove residual ointment on the surface. Skin and muscle were frozen with liquid nitrogen and pulverized. Pulverization was performed in pestle and mortor to fragment the frozen tissues samples into fine pieces. Tissue was homogenized with methanol using Mini-BeadBeater-1 (Cole-Parmer North America) for cell disruption. The homogenate was left in the sonicator for 1 hour at 25 °C and kept overnight at 4°C to allow for the complete extraction of the drug from the tissues. The homogenate was centrifuged at 2100 ± 100 rpm for 10 min. Supernatant was evaporated by sample concentrator, and the drug residue was reconstituted with rat blood. Tissue drug

concentrations are expressed as ng/g of tissue weight. To control for residual ointment or Lipoderm on the skin, ointment was applied on limbs (n=4) and immediately cleaned with ethanol-soaked gauze. Biopsies from skin were collected and analyzed for TAC and MPA concentration. The highest TAC concentrations from residual ointment that remained on the skin after wiping off were minimal (19±9ng/g for TAC and 0.8 ±0.4 µg/g for MPA) compared to the actual tissue concentrations.

### 4.3.12 Quantification of MPA in Plasma by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Fifty microliters of plasma containing an unknown concentration of MPA was added to a conical centrifugation tube, followed by 200 µL of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$). Five hundred microliter of an acetonitrile-based solution containing a deuterated internal standard (MPA-$D_3$) at a concentration of 250 ng/L were then added and the mixture was vortexed at 3000 rpm for 2 minutes. Samples then underwent centrifugation at 13,000 rpm for 3 minutes with the supernatant being poured off and collected into individual glass LCMS vials for analysis. An identical method was employed in the preparation of the calibration curve and quality control samples. Analysis was performed using a validated, reverse phased method for the detection of MPA in plasma on a Waters Micromass Quattro micro™ API mass spectrometer in positive electrospray ionization mode, utilizing multiple reaction monitoring, with an injection volume of 20 µL. The Waters 2795 Separations Module was equipped with a Atlantis dC18 column (2.1 x 20 mm, 5 µm) heated to 40° C. Analytes were effectively separated using a gradient elution consisting of an aqueous mobile phase (95% $H_2O$ / 5% MeOH) and an organic mobile phase (100% MeOH), at a flow rate of 0.4 mL per minute. In order to optimize atomization and enhance the

quality of chromatographic output, both mobile phases also contained 0.1% formic acid and 2mM ammonium acetate. Monitored parent to product mass transitions for MPA and MPA-$D_3$ were 338.2 → 207.2 and 341.2 → 210.2 m/z, respectively. Under these conditions, MPA had a retention time of 7.0 minutes. Results were shown to be linear for concentrations ranging from the lower limit of quantification (LLQ) value of 0.3 µg/ml up to concentration values as high as 15 µg/ml, with an $R^2$ value of 0.9996. (With the lower limit for $R^2$ acceptability being defined as 0.99.) Both intra- and inter-day precision were shown to be acceptable (C.V. <10% n=3) at concentrations of 0.5, 5, and 10 µg/mL [182].

### 4.3.13 Quantification of TAC in Blood by LC-Tandem Mass Spectrometry (LC-MS/MS)

Fifty microliters of blood containing an unknown concentration of tacrolimus was added to a conical centrifugation tube, followed by two hundred microliters of a solution of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) to precipitate blood proteins. Five hundred microliter of an acetonitrile containing an internal standard (Ascomycin) at a concentration of 15ng/ml was then added and the mixture was vortexed at 3000 rpm for 2 minutes in order to ensure optimal precipitation. Samples then underwent centrifugation at 13,000 rpm for 3 minutes with the supernatant being poured off and collected into LCMS vials for analysis. An identical method was employed in the preparation of the calibration curve and quality control samples. Analysis was performed using a fully validated, reverse phased method for the detection of TAC in blood on a Waters micromass Quattro micro API mass spectrometer operated in a positive electrospray ionization mode, utilizing multiple reaction monitoring, after injection of 20 µL of sample. The Waters 2795 Alliance Separations Module was equipped with a nova-pack® C18 column, 2.1 x 10 mm cartridge (Waters # 186003523) heated to 55°C. Analytes were effectively separated using

a gradient elution consisting of an aqueous mobile phase (95% $H_2O$ / 5% MeOH) and an organic mobile phase (100% MeOH), at a flow rate of 0.6 mL per minute. In order to optimize atomization and enhance the quality of chromatographic output, both mobile phases also contained 0.1% formic acid ($CH_2O_2$) and 2mM ammonium acetate. Monitored parent to product mass transitions for TAC and Ascomycin were 821.63 → 768.33 and 809 → 756 m/z, respectively. Under these conditions, TAC was found to have a retention time of 1.2 minutes. Results were shown to be linear for concentrations ranging from the lower limit of quantification (LLQ) value of 2 ng/ml up to concentration values as high as 40 ng/ml with an $R^2$ value of 0.9996. Limit of detection (LOD) was 0.1 ng/ml. Both intra- and inter-day precision were shown to be acceptable (C.V. <10% n=3) at concentrations of 4.3, 15.7, and 24.6 ng/mL[140].

### 4.3.14 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 statistical software for windows (GraphPad Software, La Jolla, CA, USA). Six animals for each group was selected to achieve adequate power with alpha = 0.05 and beta (power) = 0.8 considering 30% variability, based on previous experience and published results, and looking for 40% difference in the effect of site-specific immunosuppression. Graft survivals in each group were plotted using the Kaplan-Meier method, and the differences in the median allograft survival between groups was analyzed using the log-rank test. Data sets were checked for normality. Data is expressed as means ± standard deviation, and Student t test and/or analysis of variance (ANOVA) test was used to compare the difference between groups. Post hoc test was used to do multiple comparisons. A p value < 0.05 was considered as statistically significant. Statistically significant data were presented as follows:

*P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. Statistical tests are specifically indicated under each figure.

## 4.4 Results

### 4.4.1 Combined treatment of topical TAC and MPA applied on the allograft in conjunction with low dose of systemic TAC promotes allograft survival (>100 days).

We evaluated whether combined treatment of topical TAC and MPA applied on the allograft in conjunction with low dose systemic immunosuppression with TAC can be effective in sustaining the allograft survival in a clinically relevant model of VCA. Lew rats (recipients) underwent orthotopic hind-limb allotransplantation. Transplanted animals in different group were treated with different immunosuppressive treatments. Treatment was continued until the study's end point (Grade 3 rejection or > 100 days survival). The allograft survival data is demonstrated graphically in **Figure 30**. Macroscopic appearance of the allografts at the end point is demonstrated in **Figure 31**. Group 1 (n =5) consisted of untreated rats. The median survival time for grade 3 rejection was 8 days. Group 2 (n = 6) was treated with low dose systemic TAC therapy (0.1mg/kg/day), and the median time to grade 3 rejection was 37.5 days. Group 3 (n = 6) was treated with high dose systemic TAC therapy (1mg/kg/day), and all animals survived until the end point (> 100 days). Group 4 (n = 4) was treated with topical TAC (0.5mg/kg/day, ointment 0.03%) + MPA (16.6mg/kg/day, lipoderm 1%) therapy, and the median time to grade 3 rejection was 24 days. Group 5 (n=5) was treated with low dose systemic TAC (0.1mg/kg/day), and topical TAC + MPA therapy on contralateral non-Tx limb. The median time to grade 3 rejection in this group was 60 days. Group 6 (n=6) was treated with low dose systemic TAC (0.1mg/kg/day) and topical TAC + MPA therapy on Tx limb and had a significantly increased the allografts survival (>100 days) compared to the other groups ($p<0.05$). Group 6 vs. Group 1; P=0.0005, group 6 vs. Group 2;

P=0.001, group 6 vs. Group 3; P>0.05, group 6 vs. Group 4; P=0.001, and group 6 vs. Group 5; P=0.001. P values were calculated by log-rank (Mantel-Cox) test.

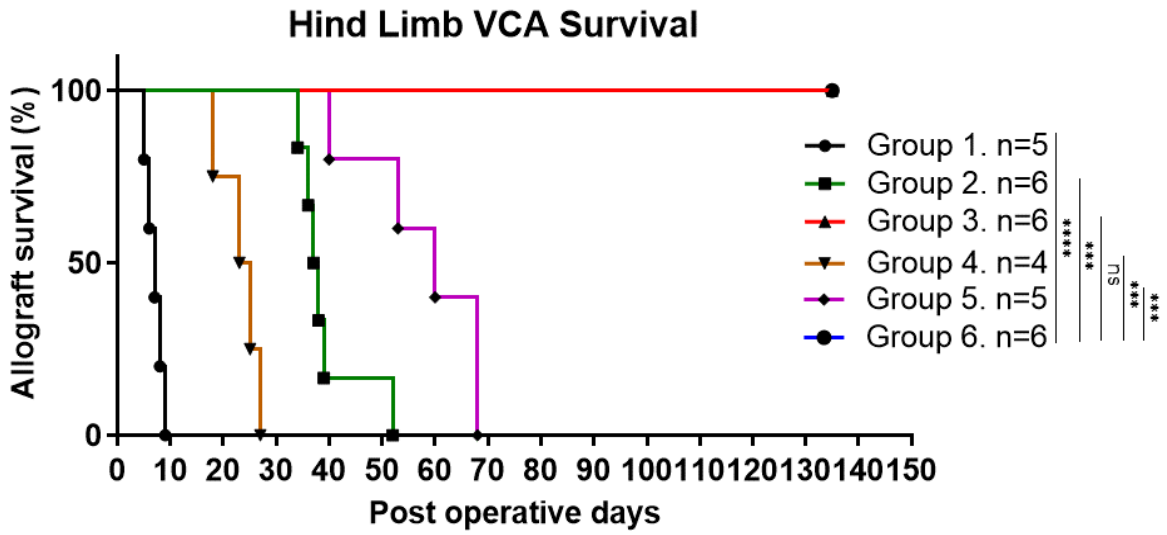



**Figure 30. Combined treatment of topical TAC and MPA applied at the allograft in conjunction with low dose of systemic TAC results in long-term survival of rat hind-limb VCA (>100 days).**

**Kaplan-Meier graft survival curves for the allograft of Brown Norway-to- Lewis orthotopic hind limb transplantation of the six groups. Group 1 (n =5) was left untreated. Group 2 (n = 6) was treated with low dose systemic TAC (0.1mg/kg/day). Group 3 (n = 6) was treated with high dose systemic TAC (1mg/kg/day). Group 4 (n = 4) was treated with topical TAC + MPA therapy. Group 5 (n=5) was treated with low dose systemic TAC (0.1mg/kg/day), and topical TAC + MPA therapy on contralateral non-Tx limb. Group 6 (n=6) was treated with low dose systemic TAC (0.1mg/kg/day) and topical TAC + MPA therapy on Tx limb. Statistical significance of p <0.05 was calculated using log-rank (Mantel-Cox) test.**

### 4.4.2 Histopathological findings are consistent with the clinical findings

To confirm the clinical evaluation of the allografts in all groups at the study's end point, skin and muscle samples were collected for histopathological evaluation. Histopathological evaluation of hind limb transplants is shown in **Figure 31**. Macroscopic appearance of the allografts from animals treated with high dose systemic TAC therapy (1mg/kg/day) (Group 3), or low dose systemic TAC and topical (TAC + MPA) therapy at Tx limb (Group 6) did not any show signs of clinical rejection but appeared with normal hair and nail growth. The skin and muscle revealed normal and intact structure without lymphatic infiltrate, edema, and necrosis. Conversely, allografts from animals that received low dose systemic TAC (Group 2), topical (TAC+MPA) therapy (Group 4), or low systemic TAC and topical (TAC+MPA) therapy at non-Tx limb (Group 5) showed typical signs of clinical rejection (Grade 3). Skin slough, epidermolysis and exudation were observed in these allografts. Histologically, grafts undergoing clinical rejection (Grade 3) revealed epidermal loss and intense and diffuse dermal inflammatory infiltrate composed of mononuclear cells.

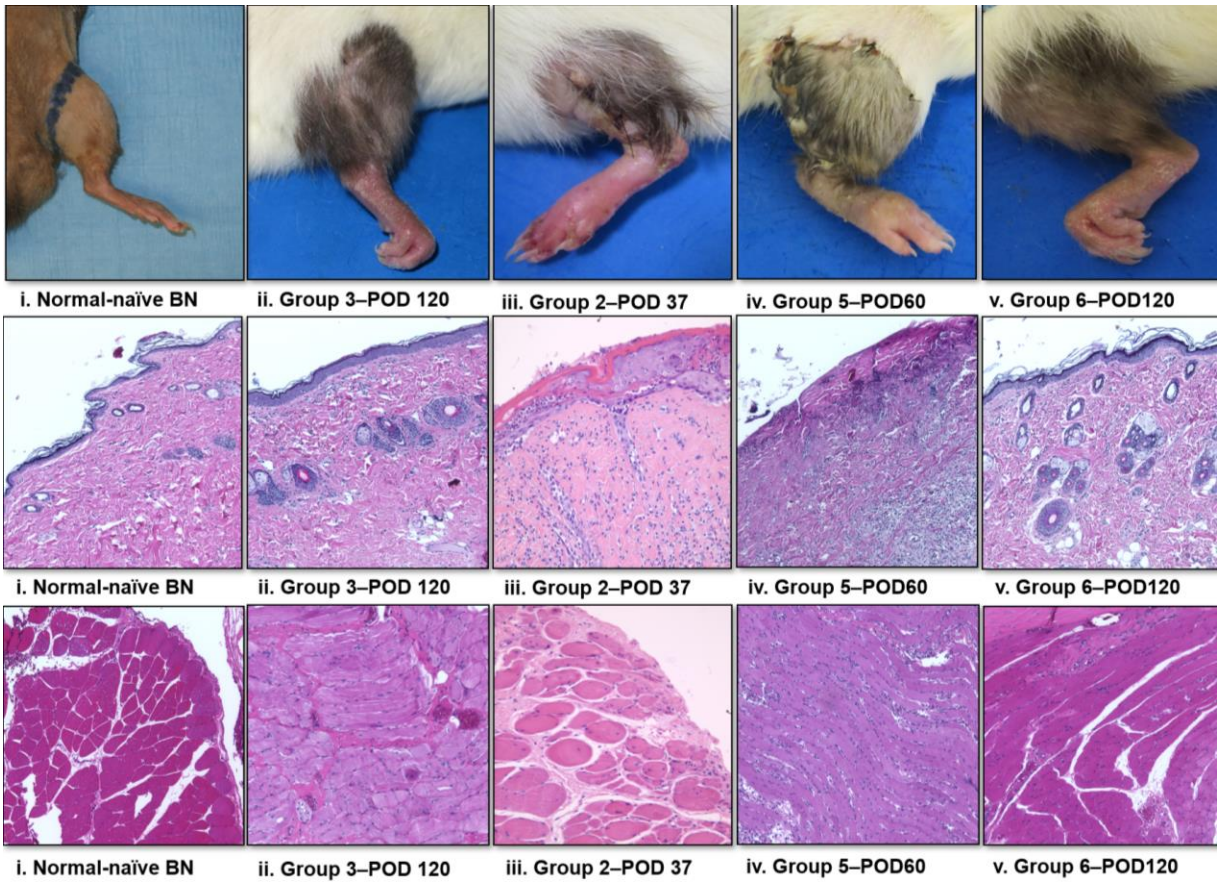



**Figure 31. Clinical and histopathological evaluation of hind limb transplants. Representative macroscopic images of hind limb allografts at the end point (Grade 3 rejection or >100 days survival). (i) Normal appearance of the naive limb. (ii) Group 3 (high STAC), day 120 post-transplant. Healthy appearance of the transplanted limb with normal hair and nail growth. (iii) Group 2 (low STAC), day 37 post-transplant. Note the signs of clinical rejection (Grade 3). Skin slough, epidermolysis and exudation were observed in these allografts. (iv) Group 5 (Low STAC+Topical therapy on non Tx limbs), 60-day post-transplant. Note the signs of clinical rejection (Grade 3). (v) Group 6 (Low STAC+Topical therapy on Tx limbs), 120-day post-transplant. Healthy appearance of the transplanted limb with normal hair and nail growth. Representative photomicrographs of the histology (H&E staining) of skin (middle panel) and gastrocnemic muscle (lower panel) from naïve brown Norway (control), and experimental groups. Rejected grafts showed severe cellular infiltrations, and necrosis while non-rejected rats showed normal and intact tissue structure with healthy basal keratinocyte layer. Magnification, 20x; scale bars, 100 µm.**

### 4.4.3 Combined treatment of topical TAC and MPA applied on the allograft in conjunction with low dose of systemic TAC provides low systemic drug exposure and high local drug exposure in the VCA graft tissues

Systemic drug exposure was evaluated in animals from all the groups by measuring trough concentrations of TAC in blood and MPA in plasma over time post-transplantation. The blood/plasma samples were collected at 24 hours after dose administration. Average weekly trough concentrations of TAC and MPA are shown in **Figure 32 (a-b)**. Average trough blood concentrations of TAC on post-operative days 7 for all groups was 10±2 ng/ml. After day 7, the average trough blood concentrations of TAC were ranged from 1 to 2.3 ng/ml in Group 2, and from 7 to 13 ng/ml in Group 3 throughout the study period, while average trough blood/plasma concentrations for groups treated with topical therapies (4, 5, and 6) were <5ng/ml for TAC (1.7±0.5, 2.6±1, and 2.8±1ng/ml) and <0.3 µg/ml for MPA (0.18±0.07, 0.14±0.05, 0.06±0.05 µg/ml) throughout the study period. Average trough blood concentrations of TAC in Group 6 was significantly lower than the values observed in Group 3 (3±1 vs. 10±2 ng/ml, respectively. ****$p < 0.0001$).

Local drug exposure was evaluated in animals from all groups by measuring drug concentrations in the tissues collected from the Tx and contralateral non-Tx limbs at the study's end point as shown in **Figure 33 (a-c) and 34 (a-c).** In groups treated with topical therapy (4, 5, and 6), concentrations of TAC and MPA in the skin, muscle, and DLNs collected from the application site were significantly higher than the values observed in the groups treated with only systemic TAC therapy (2 and 3) ($p<0.05$). Similarly, concentrations of TAC and MPA in the skin, muscle, and DLNs that are collected from the application site were significantly higher than the values observed in the skin, muscle, and DLNs that collected from the contralateral sites ($p<0.05$).

The highest TAC and MPA concentrations from residual ointment or lipoderm that remained on the skin after wiping off were minimal (19±9ng/ml and 0.8±0.4µg/ml, respectively).

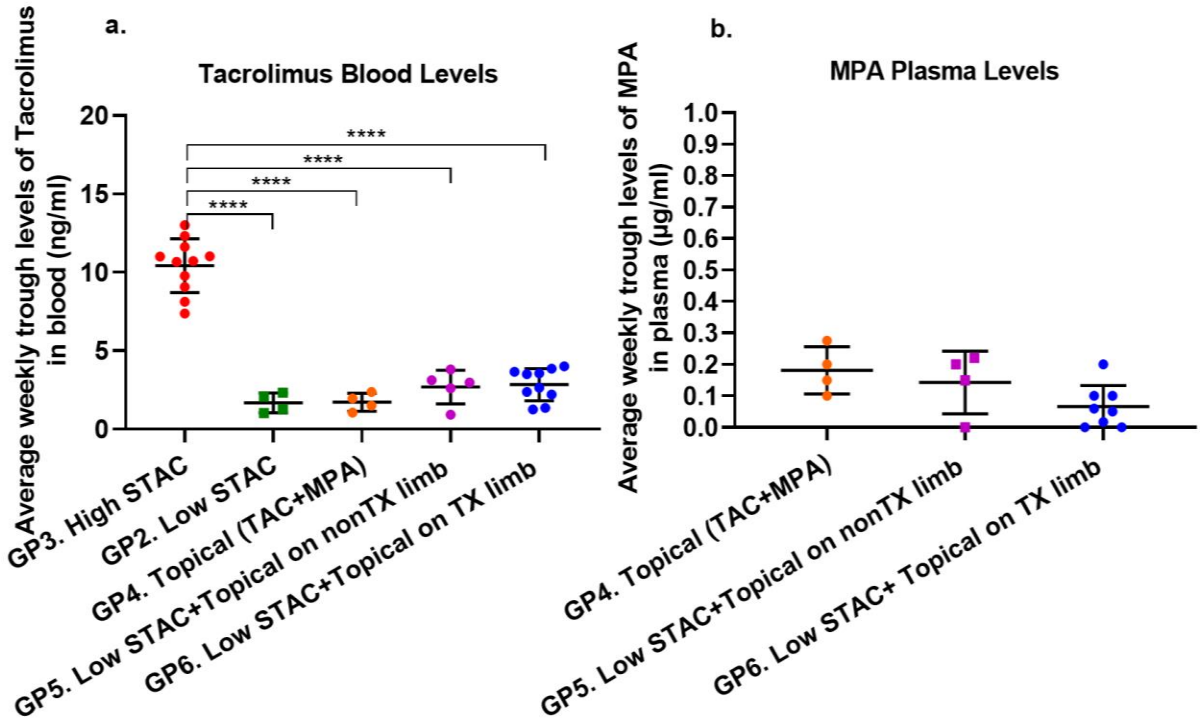
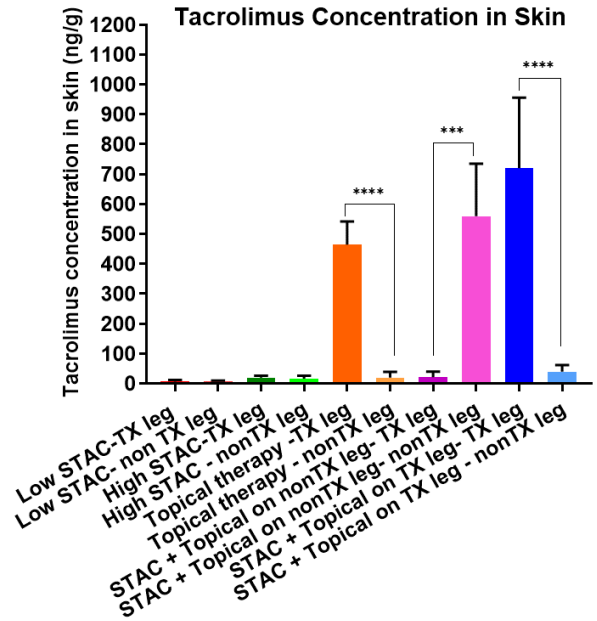
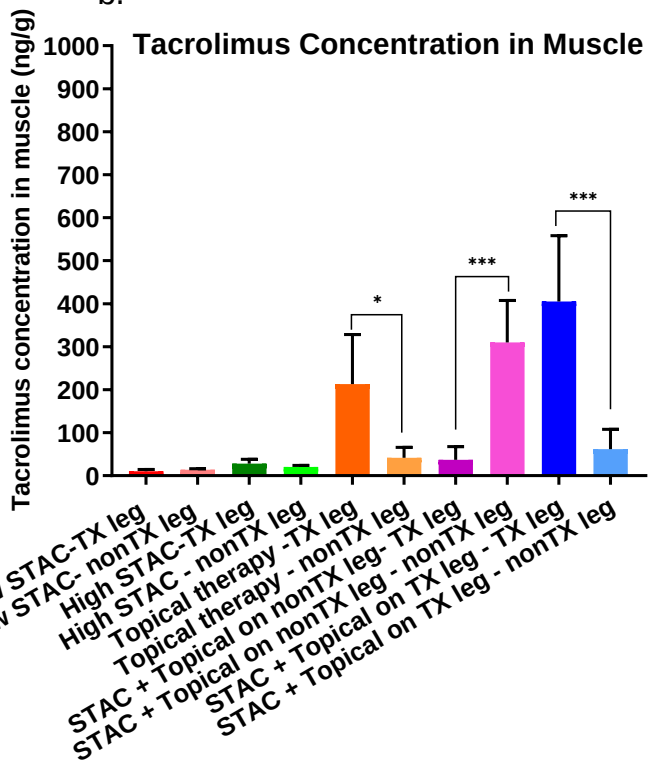



**Figure 32. Average weekly trough levels of TAC and MPA in the blood/plasma post-transplantation. (a). Average weekly trough levels of Tacrolimus in the blood (ng/ml). (b). Average weekly trough levels of MPA in the plasma (µg/ml). The blood/plasma samples were collected at 24 hours after dose administration. Statistical analyses between the groups are shown. Each data point represents the mean value of tacrolimus measurements collected weekly at the seventh day (starting from post-operative week 2, until the study end point). P values were calculated by one-way ANOVA.**

a.


Tacrolimus Concentration in Skin
Tacrolimus concentration in skin (ng/g)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
****
***
****
Low STAC-TX leg
Low STAC- non TX leg
High STAC-TX leg
High STAC - nonTX leg
Topical therapy -TX leg
Topical therapy - nonTX leg
STAC + Topical on nonTX leg- TX leg
STAC + Topical on nonTX leg- nonTX leg
STAC + Topical on TX leg- TX leg
STAC + Topical on TX leg - nonTX leg

b.


Tacrolimus Concentration in Muscle
Tacrolimus concentration in muscle (ng/g)
1000
900
800
700
600
500
400
300
200
100
0
*
***
***
Low STAC-TX leg
Low STAC- nonTX leg
High STAC-TX leg
High STAC - nonTX leg
Topical therapy -TX leg
Topical therapy - nonTX leg
STAC + Topical on nonTX leg- TX leg
STAC + Topical on nonTX leg - nonTX leg
STAC + Topical on TX leg - TX leg
STAC + Topical on TX leg - nonTX leg

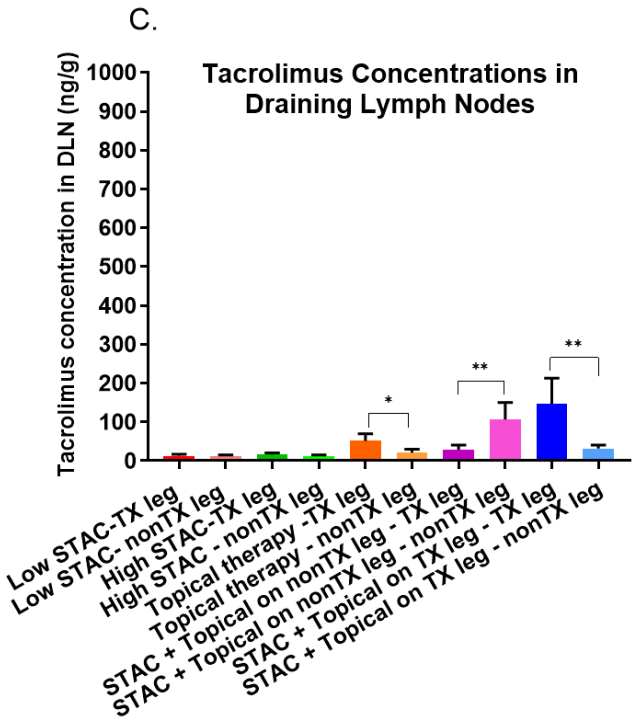
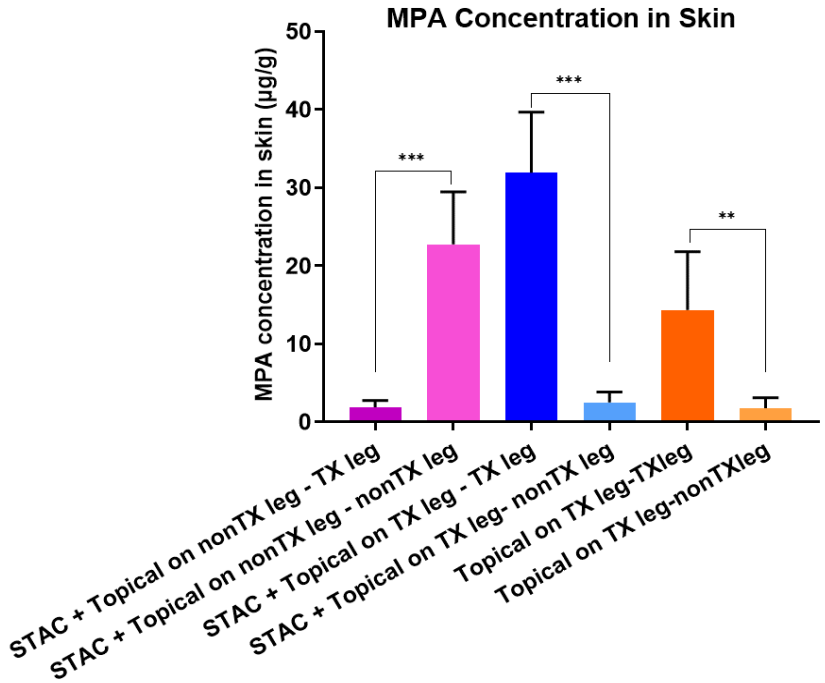
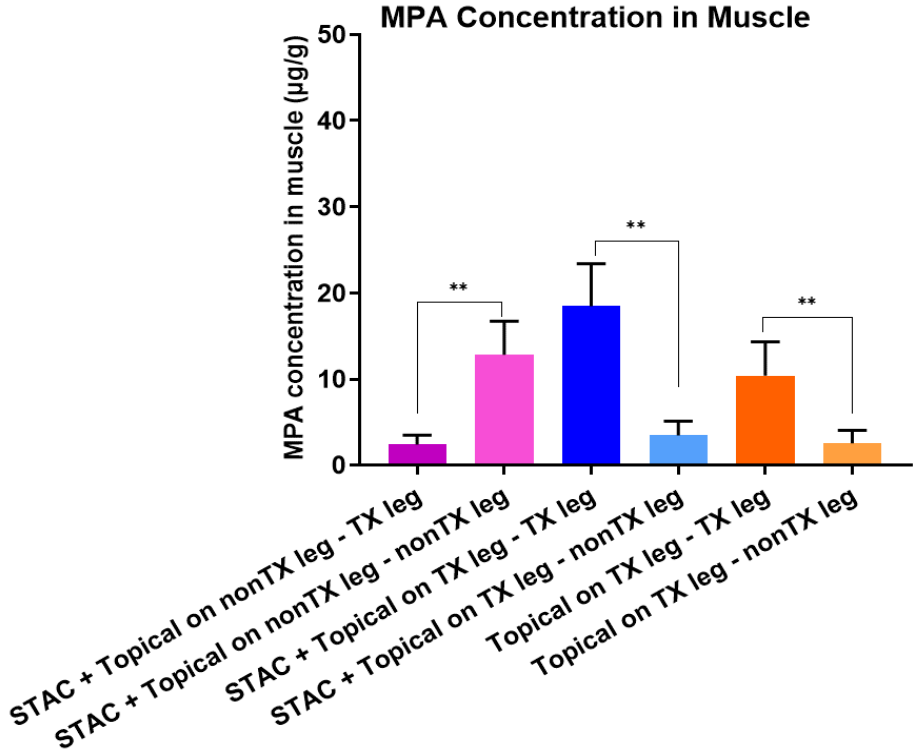



**Figure 33. Average tacrolimus concentrations (ng/g) were measured by LC-MS/MS in (a) skin, (b) muscle, and (c) DLNs collected from the Tx limbs and the other contralateral non-Tx limbs at the end point. Statistical analyses of the differences between the 2 sites of biopsy collections are shown. Data are presented as mean ± SD. Paired t test is used for comparisons within a group between the 2 sites of biopsy collections. Significant differences are indicated as * p < 0.05, ** p < 0.01, ***p < 0.001, and ****p < 0.0001.**

a.


MPA Concentration in Skin
MPA concentration in skin (µg/g)
50
40
30
20
10
0
***
***
**
STAC + Topical on nonTX leg - TX leg
STAC + Topical on nonTX leg - nonTX leg
STAC + Topical on TX leg - TX leg
STAC + Topical on TX leg- nonTX leg
Topical on TX leg-TXleg
Topical on TX leg-nonTXleg

b.


MPA Concentration in Muscle
MPA concentration in muscle (µg/g)
50
40
30
20
10
0
**
**
**
STAC + Topical on nonTX leg - TX leg
STAC + Topical on nonTX leg - nonTX leg
STAC + Topical on TX leg - TX leg
STAC + Topical on TX leg - nonTX leg
Topical on TX leg - TX leg
Topical on TX leg - nonTX leg

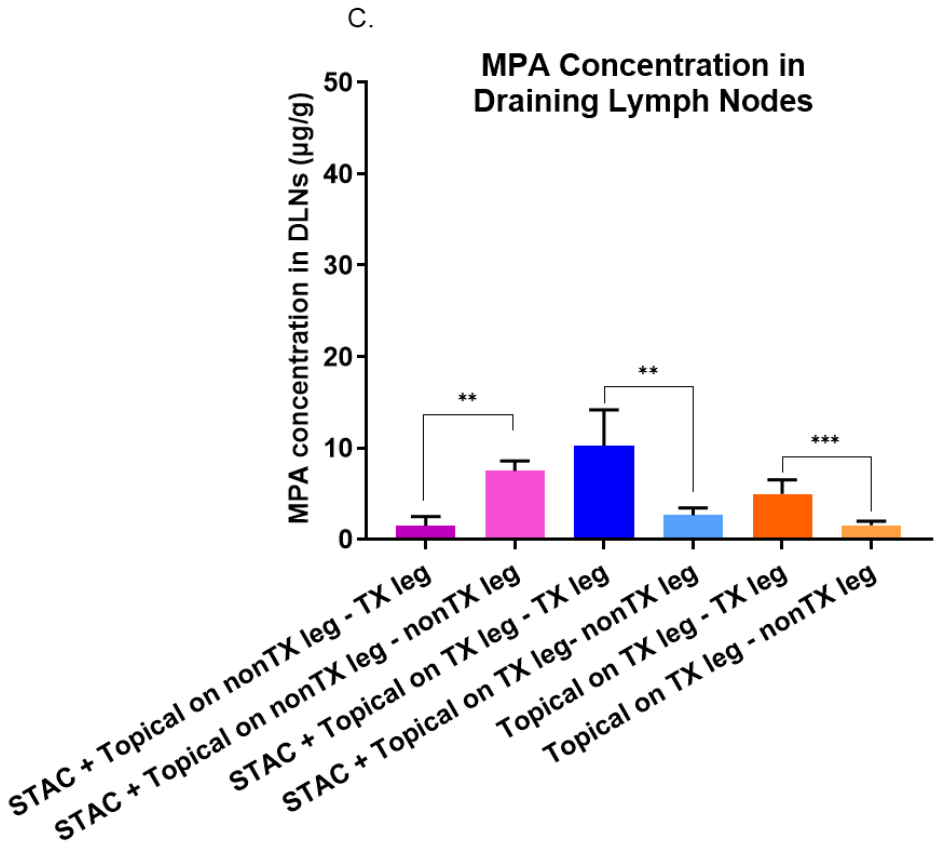



**Figure 34. Average MPA concentrations (µg/g) were measured by LC-MS/MS in skin (a), muscle (b), and DLNs (c) collected from the Tx limbs and the contralateral non-Tx limbs at the end point for groups 4, 5, and 6. Statistical analyses of the differences between the 2 sites of biopsy collections are shown. Data are presented as mean ± SD. Pairedt t test is used for comparisons within a group between the 2 sites of biopsy collections. Significant differences are indicated as * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.**

### 4.4.4 Combined treatment of topical TAC and MPA applied on the allograft in conjunction with low dose of systemic TAC does not significantly change the levels of $CD4^+CD25^+FoxP3^+$ T regulatory cells in the peripheral blood

Flow cytometry was performed to quantify levels of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) in the peripheral blood of allogeneic transplanted animals that received low dose of systemic TAC in conjunction with topical (TAC+MPA) therapy on Tx limb (Group 6), high dose systemic TAC therapy (Group 3), and low dose systemic TAC therapy (Group 2) on days 30 and 120 post-transplantation. As shown in **Figure 35 (a)**, the percent of $CD4^+CD25^+FoxP3^+$ T regulatory cells in the peripheral blood of non-rejected animals (Group 6, n=5/group) was higher as compared to the values observed in the rejected animals (Group 2, n=5) and non-rejected animals (Group 3, n=5) on post-operative day 30, but the difference was not statistically significant (7.5±3.6 vs. 4.9±1.6 and 3.9±0.2, $p>0.05$). As shown in **Figure 35 (b)**, the percent of $CD4^+CD25^+FoxP3^+$ T regulatory cells in peripheral blood of the non-rejected animals that received high dose of systemic TAC (group 3, n=6) was significantly lower than the values observed in the naïve (age-matched) animals (n=6) (3±0.3 vs. 6.4±0.6, $p=0.03$) on post-operative day 120. The percent of $CD4^+CD25^+FoxP3^+$ T regulatory cells in peripheral blood of the non-rejected animals that received low dose of systemic TAC in conjunction with topical (TAC+MPA) therapy on Tx limb was maintained between 5 and 10% and was similar to the values observed in the naïve (age-matched) animals (5.4±0.7 vs. 6.4±0.6, $p>0.05$) on post-operative day 120.

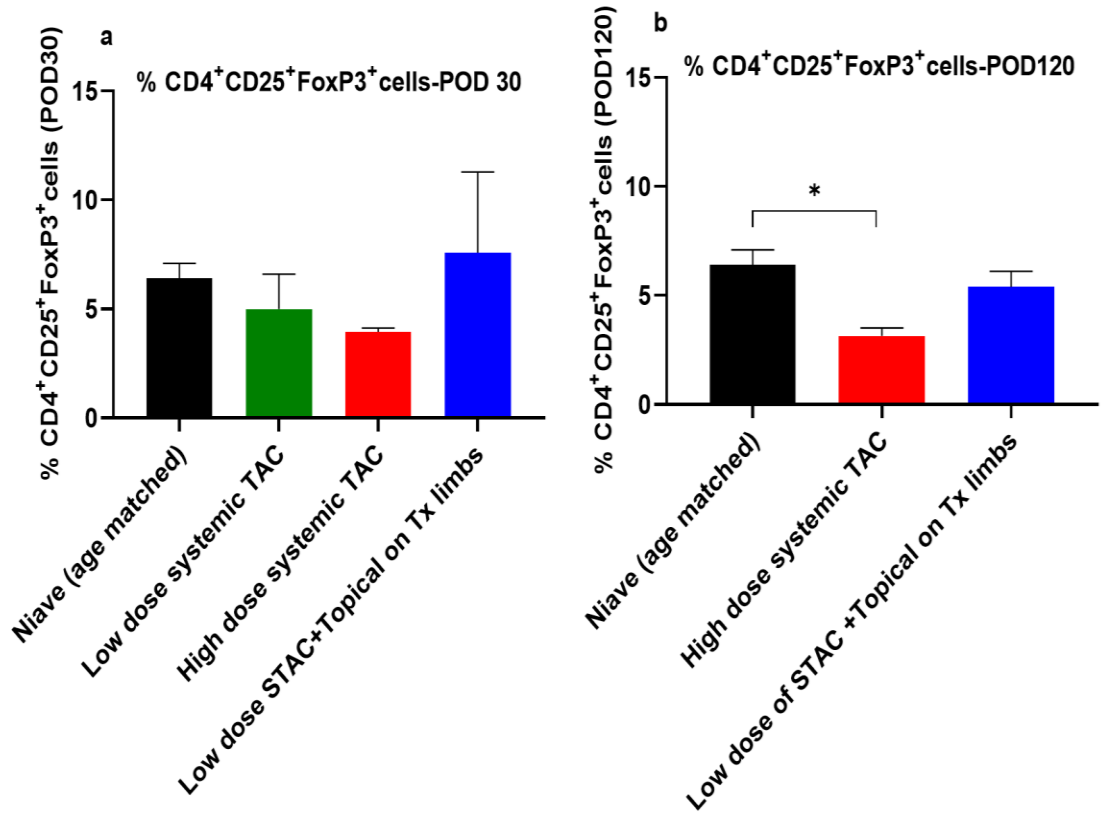



**Figure 35. Percent of CD4+CD25+FoxP3+ T regulatory cells in peripheral blood. (a). Percent of CD4+CD25+FoxP3+ T regulatory cells in peripheral blood of naïve age-matched animals (n=6) and in allogeneic transplanted animals received low dose systemic TAC therapy (Group 2, n=5), high dose systemic TAC therapy (Group 3, n=3), or low dose systemic TAC and Topical (TAC+MPA) therapy on Tx limb (Group 6, n=4) at day 30 post-transplantation. (b). Percent of CD4+CD25+FoxP3+ T regulatory cells in peripheral blood of naïve age-matched animals (n=6) and in allogeneic transplanted animals received high dose systemic TAC therapy (Group 3, n=3), or low dose systemic TAC and Topical therapy on Tx limb (Group 6, n=6) at day 120 post-transplantation. Data shown as mean±SE. P values were calculated by one-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated as * $p < 0.05$.**

### 4.4.5 Combined treatment of topical TAC and MPA in conjunction with low dose of systemic TAC does not induce in-vivo donor-specific tolerance

To test whether in vivo donor-specific tolerance had developed, animals with long-term surviving allografts (group 6) received a second challenge in the form of skin grafts on postoperative day 135. Skin grafts were harvested from Lew (self-control), WF (third party), or BN (donor) rats and transplanted into the animals with long-term allografts (n=3). As shown in **Figure 36**, Lewis skin grafts were accepted (wound healing and hair growth) as expected. WF and BN skin grafts transplanted in the back were rejected (contracture and necrosis) with median survival times 12- and 20-days post skin grafting. The allografts exhibited clinical changes after skin grafting and developed signs of grade 3 rejection by 25 days post skin grafting.

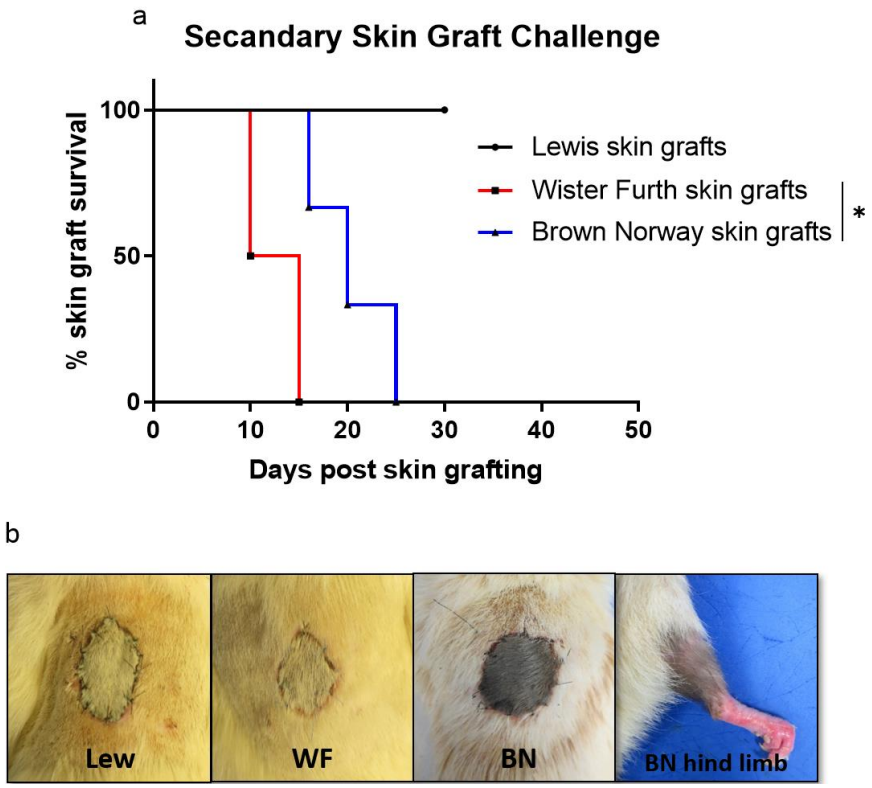



**Figure 36. Absence of donor-specific in vivo tolerance in animals with long-term survival allografts. Animals (n=3) that received low dose systemic TAC with topical therapy on Tx limbs (>100 days) were challenged with secondary full-thickness non-vascularized skin grafting from Lewis (Self-control), Wister Forth (WF), or Brown Norway (BN) rats. (a). Kaplan-Meier graft survival curves of WF and BN (donor) skin grafts. Statistical significance of p <0.05 was calculated using log-rank (Mantel-Cox) test. Significant differences are indicated by * p < 0.05. (b). Representative macroscopic pictures showing that recipient-rejected WF and BN skin grafts and hind limbs at days 12, 20 and 25 post skin grafting. Lewis-skin grafts were accepted.**

### 4.4.6 Combined treatment of topical TAC and MPA in conjunction with low dose of systemic TAC does not result in nephrotoxicity

To test whether topical delivery of TAC and MPA, in conjunction with low dose of systemic immunosuppression with TAC could cause nephrotoxicity, the kidney function as measured by the clearance of creatinine was evaluated in syngeneic Tx (Control, age matched) group (n=4), high dose systemic TAC group (Group 3, n= 4), or low dose systemic TAC in conjunction with topical therapy on Tx limbs (Group 6, n=5) at two-time points, pre-transplant (day -1) and post-transplant (day 120) during the study period as shown in **Figure 37.** No significant change was observed in the average CrCl of the syngeneic Tx (age matched) group and topical group at the two-time points, pre-transplant (day -1) and post-transplant (day 120) ($p>0.05$). The greatest deterioration in CrCl was observed in group 3 where the transplanted animals received daily systemic TAC therapy (1mg/kg/day) with a CrCL of 1.8±0.2 ml/min at day 120 post-transplant as compared to the average CrCL in the same animals at day -1 pre-transplant (**p=0.0036), and as compared to the average CrCL in the syngeneic Tx (age matched) t day 120 post-transplant (*p=0.05), and as compared to the average CrCL in the topical group at day 120 post-transplant (*p=0.05).

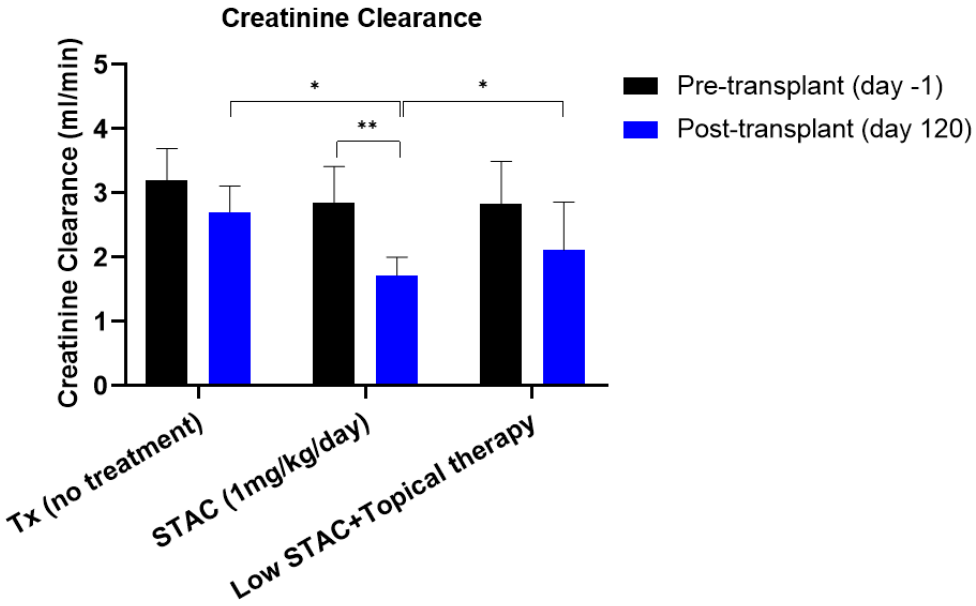



**Figure 37. Topical delivery of TAC and MPA in conjunction with low systemic TAC did not cause nephrotoxicity. Measurements of the creatinine clearance (ml/min) in transplanted animals received no treatment (Control, n=4), intraperitoneal injection of TAC in a dose of 1mg/kg/day (Group 3, n=4), or topical therapy with low systemic TAC (Group 6, n=4) at specific time points during the study period. Statistical analyses between the groups are shown. Data shown as mean+SD. Intra-group differences were evaluated by paired t test. Inter-groups differences were evaluated by one-way ANOVA with Dunnett's multiple comparisons test. Significant differences are indicated by *p<0.05, ** p < 0.01.**

### 4.4.7 Combined treatment of topical TAC and MPA in conjunction with low dose of systemic TAC does not result in hyperglycemia or diabetes

Intraperitoneal glucose tolerance test (IPGTT) was performed on POD 125 to test whether topical delivery of TAC and MPA, in conjunction with low dose of systemic immunosuppression with TAC could result in hyperglycemia or diabetes. Blood glucose levels of IPGTT are shown in **Figure 38.** All transplanted animals in the systemic TAC group (1mg/kg/day) developed prediabetes as indicated by glycemia at 120 min (IPGTT) >140 and <200 mg/dl. Transplanted

animals in the topical therapy group did not develop diabetes or prediabetes as indicated by glycemia at 120 min (IPGTT) <140 mg/dl (110.5±15.4 mg/dL). Total systemic glucose exposure in the animals that received systemic TAC therapy were significantly higher than the values observed in the animals that received no treatment or topical therapy (22631±496.7 vs. 14479±465.4 or 15323±609.7 mg.min/dL, respectively. *p<0.05).

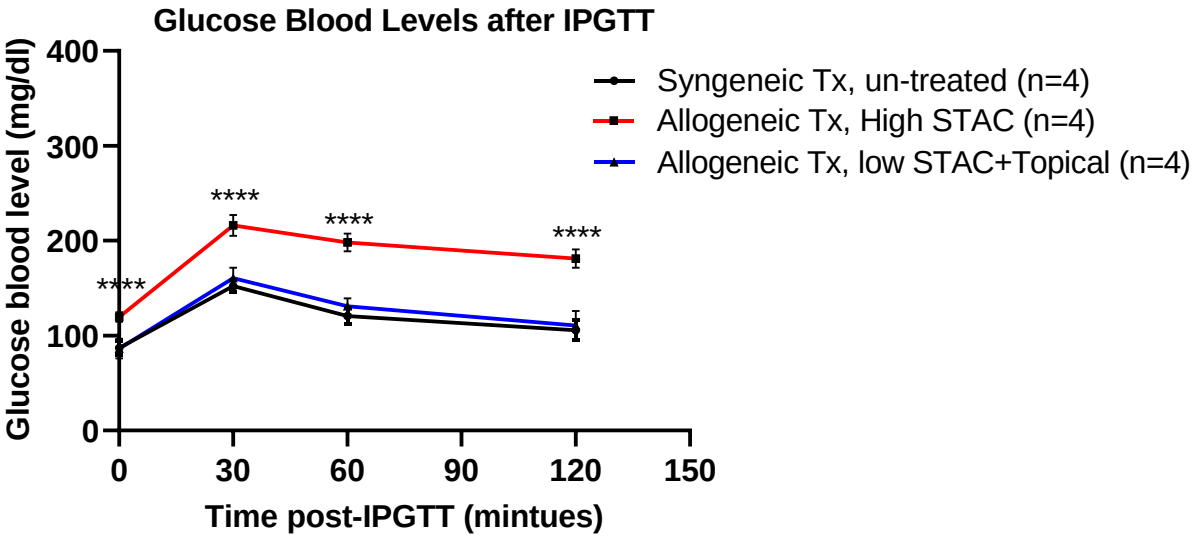



**Figure 38. Topical delivery of TAC and MPA in conjunction with low systemic TAC did not result in hyperglycemia. Intraperitoneal glucose tolerance test (mg/dl) was performed in transplanted animals received no treatment (Control, n=4), intraperitoneal injection of TAC in a dose of 1mg/kg/day (Group 3, n=4) or low dose of systemic TAC in conjunction with topical therapy on Tx limbs (Group 6, n=4) on post-operative day 125. Statistical analyses between the groups are shown. Data shown as mean+SD. P values were calculated by repeated measure ANOVA. Significant differences are indicated by **** p < 0.0001.**

### 4.4.8 Combined treatment of topical TAC and MPA in conjunction with low dose of systemic TAC does not cause significant change in the body weight

We evaluated whether topical delivery of TAC and MPA, in conjunction with low dose of systemic TAC could induce systemic toxicity, the percent change in body weight was evaluated for animals in all groups as shown in **Figure 39**. In the first week post-transplant, the average body weight of the animals in the groups were 310 ± 22 g (7 ± 3% decrease as compared to the initial body weights). The average body weight of the animals that received low dose systemic TAC + topical therapy on Tx limbs (Group 6, n=4) were 281± 4 g (5 ± 2% decrease as compared to the initial body weights) and gradually increased over time to nearly achieve the initial body weights on day 14 post-transplant, while the average body weight of the animals that received high dose of systemic TAC therapy (Group 3, n=4) continue to decline until day 14 post-transplant, and then slowly increased over time. After 30 days, the average body weights of animals in group 6 was 332±16g (12±5% increase as compared to the initial body weights), while the average body weights of animals in group 3 was 307±19g (5±5% increase as compared to the initial body weights). At the end point, animals in all groups had significant increase in the body weight as compared to the initial body weight (<0.05). However, control animals (syngeneic, un-treated) had the highest increase of their body weight, followed by animals in group 6, and animals in group 3 had the smallest increase of their body weight when all groups are compared at the end point (<0.05).

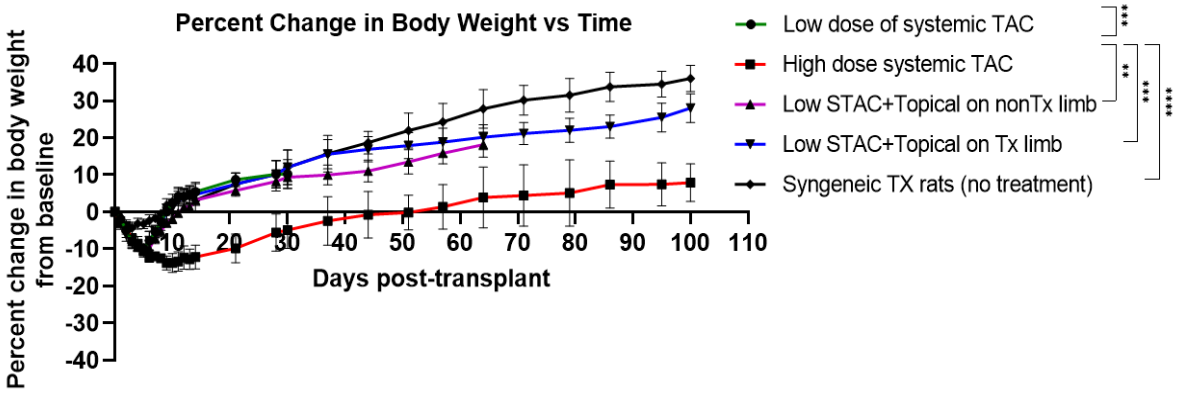



**Figure 39. Measurements of the body weight in the syngeneic transplanted (age matched) animals left un-treated (n=4), allogeneic transplanted animals received low dose systemic TAC therapy (0.1mg/kg/day) (Group 2, n=4), high dose systemic TAC therapy (1mg/kg/day) (Group 3, n=4), low dose systemic TAC and topical therapy on contralateral nonTx limb (Group 5, n=4), and low dose systemic TAC and topical therapy on Tx limb (Group 6, n=4) at different time points during the study. Statistical analyses between the groups are shown. Data shown as mean+SD. Intra-groups differences (initial body weight vs body weight at the end point) were evalauated by paried t test and inter-groups differences at the end point were evalauated by one-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by ** p < 0.01, *** p < 0.001, and **** p < 0.0001.**

## 4.5 Discussion

Use of topical immunosuppressants has been reported in experimental and clinical VCA, and the results support the concept of local immunosuppression, and suggest the potential benefits of topical application of immunosuppressive drugs in VCA. Previous studies have shown that topical immunosuppressants successfully prevented skin rejection in experimental and clinical face and hand transplants, but it may not be adequate to sustain the whole allograft. Therapeutic combination of topical immunosuppressants (TAC and MPA) with low dose STAC and its systemic and local exposure and synergistic benefits have not been studied in VCA. This study evaluates whether combined treatment of topical TAC and MPA applied at the transplant site with low dose systemic immunosuppression with TAC can be effective in sustaining VCA graft survival and in reducing systemic morbidity. The pharmacokinetic studies in chapter 2 and 3 show that topical application of TAC (Protopic®, 0.03%) once daily at a dose of 0.5mg/kg and MPA (Lipoderm, 1%) once daily at a dose of 16.6mg/kg were effective in achieving high local tissue concentrations for local effects with low systemic exposures. In the current study, TAC and MPA formulations were applied at different times in order to minimize the risk of transdermal drug interaction (e.g. alter release kinetics). Tacrolimus ointment (evening dose) was applied 12 hours after MPA in Lipoderm application (morning dose). Blood/plasma samples were collected 24 hours after dose administration of each drug for trough level measurement.

Experimentally, allogeneic orthotopic rat hind limb allotransplant is a well-established model for VCA in small animals. Lew and BN rats are a complete MHC mismatch resembling the clinical situation. Transplanted animals were treated with different immunosuppressive regimens. Allografts survival was evaluated and compared to assess the efficacy of the treatment modality. Concentrations of TAC and MPA in the blood and the allograft were measured to determine the

effective concentrations to prevent the rejection. Glucose blood levels and creatinine clearance were measured to assess the systemic toxicity. The immunomodulatory effects of TAC and MPA were studied to identify the mechanism that is responsible for the allograft survival.

Following transplantation, all transplanted animals received systemic TAC dose (1 mg/kg/day) that is known to maintain the allograft [151, 219]. High levels of systemic immunosuppression are required to inhibit the overwhelming immune response early following transplantation and to allow the topical therapy to exert its local immunosuppressive effect. In group 2 (low STAC), transplanted animals rejected their allografts in an average 37 days after dropping the doses from 1mg/kg/day to 0.1 mg/kg/day. In VCA, its known that TAC trough levels <5 ng/mL are associated with a higher risk for acute rejection [220]. These low systemic levels allow to evaluate the efficacy of the topical therapy in sustaining the allograft survival. In group 3 (high dose of STAC, 1mg/kg/day), transplanted animals maintained their allografts to the end point (>100 days). TAC concentrations in the blood were ranged between 7-13 ng/ml, and in the local tissues were 19±7 ng/g in skin, 24±8 ng/g in muscle, and 22±7 ng/g in DLNs.

The best outcomes were achieved in the transplanted animals treated with low dose systemic TAC in conjunction with topical (TAC+MPA) therapy applied on transplanted limbs (group 6). The animals maintained their allografts for >100 days without local or systemic complications in agreement with two previous studies in which topical therapy was effective in preventing acute skin rejection episodes [207, 208]. Applying topical (TAC+MPA) therapy on the contralateral non-transplanted limbs (group 5) was less effective, suggesting that the immunosuppressive effect is exerted locally by TAC and MPA at the transplant site with minimal influence on the systemic drug levels. Furthermore, low “sub-therapeutic” blood levels of TAC

were observed during the study duration indicating that the high blood levels of TAC are not necessary for allograft survival.

Low dose of systemic TAC (group 2) or topical (TAC+MPA) therapy alone (group 4) were less effective when administered separately, indicating that the high loco-regional concentrations of TAC and MPA at the allograft are necessary for the allograft survival in conjunction with the low systemic levels. This confirms the additive benefit of using topical (TAC+MPA) therapy applied at the allograft in conjunction with low systemic immunosuppression with TAC in preventing skin rejection and prolonging allograft survival as compared with low dose of systemic TAC or topical (TAC+MPA) therapy alone.

Once daily combined treatment of topical (TAC+MPA) therapy applied at the allograft in conjunction with low dose of systemic immunosuppression with TAC effectively inhibit both local (donor allograft) and systemic (recipient) immune response and prevent the allograft rejection. This finding has been confirmed histologically by the absence of any sign of rejection in the skin and muscle. Combining TAC and MPA exhibited profound inhibitory effect on the T cells proliferation as indicated by the significantly decreased lymphocyte infiltration in the skin. The concentrations of TAC and MPA were significantly higher in the allograft particularly the skin, when compared to the concentrations observed in the blood or plasma (TAC: 250-fold higher; MPA 480-fold higher). High concentrations of TAC and MPA observed in the allograft skin, muscle and DLNs are desirable, because these are the targeted tissues for the pharmacologic effects. These findings indicate that long-lasting high loco-regional concentrations of TAC and MPA in the allograft were effective in inhibiting the allo-immune response and maintaining the survival of allograft.

Animals that received topical (TAC+MPA) therapy on the contralateral non-transplanted limbs in conjunction with low dose of systemic TAC (group 5) rejected their allograft by day 60. The treatment results in significantly higher locoregional concentrations of TAC and MPA in the non-transplanted limb particularly the skin, when compared to the concentrations observed in the blood and/or plasma (TAC: 200-fold higher; MPA: 160-fold higher). The concentrations of TAC and MPA observed in the allograft particularly the skin was significantly lower than the non-transplanted limb (TAC:25-fold lower; MPA:12-fold lower). The low concentrations in the allograft tissues (TAC: 22±18ng/g for skin, 36±13ng/g for muscle, 28±11ng/g for DLNs; MPA: 2±1µg/g for skin, 2.4±1.1µg/g for muscle, 7.5±1.1µg/g for DLNs) along with the low blood levels of TAC (2.6±1ng/ml) were insufficient to maintain the allograft survival over a prolonged time. This indicates that the effective concentrations that should be achieved in the allograft tissues to prevent the rejection should be higher than these concentrations. However, these concentrations are higher than the concentrations that observed in group 3 (high dose of STAC, 1mg/kg/day). These findings indicate that the allograft survival was maintained by the long-lasting high locoregional concentrations of TAC and MPA in the allograft tissues along with the low systemic levels of TAC. Concentrations of TAC and MPA in the allograft tissues were significantly higher, when the topical formulations were daily applied on the allografts. Therefore, topical application of TAC (Protopic®, 0.03%) at a dose of 0.5mg/kg and MPA (Lipoderm, 1%) at a dose of 16.6mg/kg once every 2 days can be effective in reducing the local drug accumulation associated with daily application and in achieving and maintaining the effective therapeutic tissue concentrations for local effects with low systemic exposures.

The effect of transplantation (surgical inflammation) on the drug absorption through the skin was studied. We compared the blood levels and tissue concentrations of TAC and MPA

between animals that received topical therapy either on the transplanted limb (group 6) or on the non-transplanted limb with intact healthy skin (group 5). The systemic exposure (troughs) and local tissue concentrations of TAC and MPA at the application site were higher when the topical formulations were applied on the transplanted limbs. However, the difference was not significant between the two groups ($p>0.05$). This indicates that transplantation (surgical inflammation) has a minimal effect on the systemic drug absorption and exposure.

Our immunological results showed that there was no central or peripheral tolerance development in the animals with the surviving allografts (>100 days). Studies have shown that graft survival was accompanied by the continuous elevation of $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory (Tregs) levels in experimental VCA [221]. Tregs mediate immune hyporesponsiveness via expression of CTLA-4 which interacts with dendritic cells (DCs) and induces these DCs to adopt a suppressive phenotype, and thereby make them un-able to activate naïve T cells. T regs also secret suppressive cytokines such as IL-10 and TGF-β [222]. In our study, we found that combined treatment of low dose systemic TAC in conjunction with topical (TAC+MPA) therapy applied on Tx limbs did not significantly change the levels of Tregs in the peripheral blood and these levels were similar to the values observed in the naïve (age-matched) animals (5-10% of all T cells) [223]. Whereas high dose of systemic TAC significantly reduced the levels of Tregs in the peripheral blood as compared to the values observed in the naïve (age-matched) animals. The skin grafting data shows the absence of donor-specific in-vivo tolerance in the animals with surviving allografts >100 days. This indicates that the allograft survival was not related to the induction of donor-specific tolerance but due to the long-lasting high loco-regional concentrations of TAC and MPA in the allograft.

Another goal of our current treatment strategy is to reduce the toxicity of immunosuppressive drugs. TAC is associated with long-term side effects. These include nephrotoxicity and metabolic complications [64, 224]. We evaluated the long-term systemic toxicity of our treatment strategy and compared it to the standard systemic immunotherapy with TAC. Nephrotoxicity is a severe complication following organ transplantation and has been reported to occur in 26% of hand transplant patient. Use of TAC is a major risk factor for nephrotoxicity. The effects of TAC on kidney have been evaluated in preclinical and clinical transplantation studies. TAC was nephrotoxic in animals and humans. TAC decreased renal function and increased histologic damage [225, 226]. TAC concentration toxicity relationship has been established [227-230]. Therefore, maintaining the concentrations within the targeted therapeutic range can reduce the risk of nephrotoxicity [230]. The results show that transplanted animals that received combined treatment of low dose systemic TAC in conjunction with topical (TAC+MPA) therapy did not show sign of nephrotoxicity as indicated by the normal creatinine clearance with an estimated mean trough levels of TAC <5 ng/ml. Whereas animals that received high dose of systemic TAC (1mg/kg/day) where the treatment resulted in estimated trough mean levels of TAC 10±2 ng/ml showed signs of nephrotoxicity as indicated by the significant decrease in the creatinine clearance rates to 50% of the values observed in the animals that received did not receive any treatment ($p<0.05$). Our results are consistent with other studies [225] and confirmed our hypothesis that topical therapy allows sustained allograft survival when combined with low systemic therapy and therefore lowers the risk of systemic toxicity.

Diabetes is a severe complication following organ transplantation and has been reported to occur in 40% of the hand transplant patients. Use of TAC is a major risk factor for diabetes. The results show that combined treatment of low dose systemic TAC in conjunction with topical

(TAC+MPA) therapy did not result in hyperglycemia or diabetes, where the treatment resulted in estimated trough mean levels of TAC between 1-4 ng/ml. Average glucose levels were between 80 - 100 mg/dL, and ≤ 100 mg/dl at 120 min (IPGTT). While daily high dose of systemic TAC (1mg/kg/day) induced diabetes with an estimated mean trough levels of TAC of 10±2 ng/ml. This indicates that TAC affects the metabolic activity of the pancreas and this effect depends on the dose and the duration of exposure [231, 232]. These results are consistent with other studies where prolonged treatment with high doses of systemic TAC (orally or subcutaneously, at least 1 mg/kg/day) developed diabetes where the treatment resulted in estimated trough mean levels of TAC of 8-10 ng/ml [232-235]. Prolonged treatment with systemic TAC (0.1mg/kg, intraperitoneal injection, twice a day) where the treatment resulted in estimated trough mean levels of TAC of 8.3±1.8 ng/ml results in diabetes. [236]. The mechanism of how TAC induces hyperglycemia or diabetes is currently under investigation, but studies have shown that TAC inhibits insulin secretion and action [237, 238]. This explains the elevated glucose levels, impaired glucose tolerance, and reduced body weight that were observed in the animals receiving daily high dose of systemic TAC. However, animals treated with topical therapy in conjunction with low systemic TAC had significantly higher increase of their body weights when all groups are compared at the end point.

This work has shown that once-daily combined treatment of topical immunotherapy (TAC+MPA) with low dose of systemic immunotherapy sustained allograft survival by sustained site-specific immunosuppression at the allograft site and reduced overall systemic drug exposure and associated systemic side effects (nephrotoxicity or diabetogenicity). We conclude that combined treatment of topical TAC and MPA applied at the transplant site with low dose of STAC can be an effective therapeutic strategy to sustain VCA graft survival and reduce systemic

morbidity. The allograft survival was not related to the induction of donor-specific tolerance but to the long-lasting high loco-regional concentrations of TAC and MPA in the allograft. Further studies should be performed to determine the minimum effective therapeutic concentrations that should be achieved in the VCA graft tissues particularly skin in order to prevent the rejection. The dosing regimen of topical immunosuppressive drugs can be further modified to achieve and maintain the effective therapeutic concentrations in the VCA graft tissues.

# 5.0 Tacrolimus-Eluting Disk Platform in the Allograft Enable Vascularized Composite Allograft Survival Without Systemic Toxicity

## 5.1 Abstract

Poor adherence to immunosuppressive therapy is a major cause of graft rejection and graft loss. Chronic systemic immunosuppression however leads to metabolic, infectious, or neoplastic complications. Our goal was to develop a site-specific immunosuppressive strategy that promotes VCA allograft survival and minimizes the risk of systemic side effects of immunosuppressive drugs using a novel technology of drug-eluting biomaterials that is inserted in the allograft. Tacrolimus loaded polycaprolactone (TAC-PCL) disks were prepared and tested for their efficacy in sustaining the survival of VCA graft via site-specific immunosuppression. Brown Norway-to-Lewis rat hind limb transplantations were performed; the animals received one TAC disk either in the transplanted (DTx) with or without lymphadenectomy, or in the contralateral non-transplanted (DnonTx) limb. Blood and allograft levels of TAC were measured using LC-MS/MS. Blood glucose levels and creatinine clearance were measured to assess systemic toxicity. The immunomodulatory effect of TAC was assessed by flow cytometry and mixed lymphocyte reaction. Animals that received TAC disks in Tx limbs achieved long-term allograft survival (>150 days) without signs of metabolic, infectious, or neoplastic complications. In these animals, TAC levels in blood were low but stable between 2 to 5 ng/ml for nearly 100 days. After this, the levels dropped to <2ng/ml. Long-lasting high concentrations of TAC were achieved in the allografts and draining lymphatic nodes (DLNs). Animals that underwent lymphadenectomy rejected their allograft by 175 days. Animals that received DnonTx rejected their allografts by 70 days. Systemic lymphocyte proliferative response appeared unaffected with local TAC. Controlled delivery of TAC directly to the allograft and DLN (with a single TAC disk) over a prolonged period effectively inhibits rejection and significantly prolongs VCA allograft survival, while mitigating the

complications of systemic immunosuppression. There was a profound survival benefit of delivering TAC within the allograft as compared to a remote site.

## 5.2 Introduction

Widespread clinical applicability of vascularized composite allotransplantation (VCA) has been limited by high-dose, systemic, multi-drug immunosuppression [239]. Chronic systemic immunosuppression leads to metabolic, infectious, or neoplastic complications [240]. Compared to solid organ recipients, a higher incidence of infections and metabolic complications occur in VCA patients [241]. Poor adherence to the immunosuppressive therapy is a major cause of graft rejection [242]. There is an immediate need for strategies that promote VCA allograft survival and minimize the risk of systemic side effects of the immunosuppressive drugs.

Immunosuppression at the transplant site may be a promising strategy to prevent the rejection and to minimize the risk of systemic side effects of systemic immunosuppression [107]. Many of the priming mechanisms that lead to the formation of the alloimmune response occur within the donor graft [243]. Lymph nodes (LNs) draining the allograft are important sites for the priming and activation of alloreactive T cells, and they also play an important role in the modulation of alloimmune response [108, 244, 245]. Therefore, site specific delivery of immunosuppressive drugs to the allograft and draining lymph nodes (DLNs) could provide efficient immunosuppression to prevent rejection, while limiting the complications of systemic immunosuppression [94, 104, 246].

Vascularized composite allograft exemplified by hand and face allografts are readily accessible for site- specific delivery of immunosuppressive drugs, visual monitoring, and direct therapeutic intervention [94]. These include the use of topical formulations [104, 207], microspheres [247], nano-micelles [248], intra-graft injections [249], thermoresponsive nanogels [250], and subcutaneous administered enzyme responsive hydrogels [251]. While promising data was obtained, drawbacks for practical applications include the need for high patient compliance

and limited skin penetration of the topically applied drugs, limited drug loading capacity, uncontrolled burst release upon injection and high blood levels, failure to demonstrate controlled 'on demand,' or 'on cue' drug release in vivo, inflammatory response or foreign body reactions, and inability to remove these drug delivery systems when systemic or local complications are observed.

We have previously developed a biodegradable disk containing tacrolimus-loaded double-walled microspheres (40mg) that could release TAC in a controlled manner over a prolonged time period. The disks were inserted in the allografts and could maintain the allograft survival (100%) for 180 days [106]. However, because of the prolonged high systemic TAC exposure, it was not clear whether local or systemic immunosuppression was responsible for preventing the allograft rejection. Therefore, it is unclear whether rejection can be inhibited by local TAC, or whether effective immunosuppression is only possible by systemic administration of TAC. In this study, we have developed a drug-eluting biomaterial that is made from PCL and TAC that can be inserted in the allograft and can release TAC directly into the allograft over a prolonged period with lower initial burst and overall systemic TAC exposure. We evaluated the efficacy of this delivery system in sustaining VCA allograft survival without systemic side effects. We hypothesized that sustained loco-regional delivery of TAC directly to the allograft and DLNs could provide high loco-regional drug concentrations to efficiently inhibit the alloimmune response, prevent the rejection, and significantly prolong the survival of VCA allografts with decreased systemic drug availability and toxicity.

## 5.3 Materials and Methods

### 5.3.1 Chemicals

Tacrolimus was purchased from LC Laboratories, Woburn, MA, USA. Polycaprolactone pellets (Mn=90.000), phosphate buffered solution, cremephor EL, sigmacoate, and solvents (Dichloromethane; ethanol; methanol), ACS Analytical Grade, were purchased from Sigma–Aldrich, St. Louis, MO, USA. Antibodies in various fluorochrome combinations were purchased from BD Biosciences (San Diego, CA).

### 5.3.2 Preparation of TAC - PCL Disks

TAC loaded PCL disks were prepared in our laboratory by a solvent casting method [252]. PCL (10% w/v) was dissolved in a mixture of dichloromethane and ethanol (2:1) followed by addition of TAC (5% w/w) under constant stirring for 1 hour, and then stirred in ultrasonic water bath for 10 min to get a homogenous mixture. The polymeric mixture was added into the mold. The mixture was dried at room temperature overnight. The disks were washed with phosphate buffered solution (PH 7.4) and freeze dried to remove the water molecules.

### 5.3.3 Morphology, Drug Loading and Encapsulation Efficiency

In addition to the macroscopic (visual) examination, scanning electron microscopy was used to assess the microstructure of the disks and the pore size. The prepared TAC loaded PCL disks were sputter-coated with gold/palladium and imaged using standard scanning electron

microscopy (SEM) methods (JSM-6330F, JEOL USA). TAC loaded PCL disk was dissolved in 1 ml dichloromethane under constant stirring for 30 mintues. Nine milliliters of methanol were added to the solution. Air was introduced to evaporate the organic solvents, and the drug residue was reconstituted with blood and analyzed for TAC content using LC-MS/MS. Drug loading content (DL) and encapsulation efficiency (EE) was determined using the following equations. DL (%) = (Actual TAC mass in disk/disk mass) × 100%. EE (%) = (Actual mass of TAC/theoretical mass of TAC) × 100%.

### 5.3.4 In-Vitro Degradation and Porosity

The degradation rate of PCL disks was analyzed at 37°C in phosphate buffered solution as described [253]. Disks (1cm in diameter, 0.5cm thick) were prepared from an unloaded, and 5 mg TAC loaded polymeric mixture. The initial weight (Wi) of the dry disk was measured. Disks were subjected to the rotary shaker incubation at a rotor speed of 100 rpm in a 15-ml PBS at PH=7.4. At each time point, the disks were removed from the solution, and the weight was measured before and after drying under vacuum at 37°C overnight. Degradation rate and porosity were calculated according to the following equations: Degradation rate or mass loss (%) = 100(Wi-Wd/Wi). Porosity (%) = (Ww-Wd)/Pw x $\mu R^2$ Tx100% where Ww, Wd, and Wi represent the wet weight, dry weight, and initial weight of the disks, respectively. Pw is the density of water ($g/cm^3$), T is the thickness (cm), R is the radius (cm).

### 5.3.5 In-Vitro Release Kinetics

TAC loaded PCL disks were incubated in 50-ml PBS at PH=7.4 containing 0.5% cremophore to improve the solubility of TAC in the aqueous solution. The closed glass container coated with sigmacote was immersed in a shaking water bath at 37°C with a rotor speed: 100 rpm. A sample of 1 ml medium was taken daily for the first week, and then weekly for one month. An equivalent volume of fresh medium (1ml) was immediately replaced in order to maintain the sink conditions. Samples are analyzed for TAC content using LC-MS/MS. The experiments were performed 3 times, and the results were expressed as mean ± SD. The curve of percent drug released Vs time was plotted. Cumulative amount released (%) = Mt/Mtotal × 100%. Mt is the amount of TAC released from the PCL disk at time t and Mtotal is the total amount of TAC loaded in the disk.

### 5.3.6 Animals

All animal experiments were performed under a protocol approved by the institutional animal care and use committee (IACUC), University of Pittsburgh. Male Lewis rats (recipients) and male Brown Norway rats (donors) aged 8 to 10 weeks, weighing 250 to 300g were purchased from Charles River Laboratories (Horsham, PA). This combination represents a full major histocompatibility complex mismatch. Veterinary care of the animals was provided in the specific pathogen–free animal facility of the University of Pittsburgh.

### 5.3.7 Assessing In-Vivo Release, Pharmacokinetics, and Tissues Distribution of Single TAC-Eluting Disk Subcutaneously Implanted in Hind Limb of Rats

Lewis rats (n=6) received a single TAC disk subcutaneously implanted in the medial thigh. Blood samples (300µl) were collected from the tail vein at specific time points post-disk implantation for TAC level measurement. At the endpoint (blood drug levels fall below LLQ 2ng/ml), biopsies from skin, muscle, sciatic nerve, and DLNs were collected from the disk implanted limbs and the contralateral limbs for TAC concentration measurement using LC-MS/MS.

### 5.3.8 Assessing Efficacy of Site-Specific Immunosuppression using Single TAC-Eluting Disk within the Allograft in Sustaining Allograft Survival in Rat Orthotopic Hind Limb Allotransplantation

Lewis rats (recipients) underwent orthotopic hind-limb allotransplantation. The hind limbs were received from full MHC mismatched Brown Norway rats (donors). Lewis rats were allocated into 5 groups. Animals received no treatment (Group 1), systemic TAC (STAC) at a dose of 1mg/kg/day (Group 2), one TAC disk either in the contralateral non-transplanted (DnonTx) limbs (Group 3), or in the transplanted (DTx) limbs (Group 4). Animals in group 5 received one TAC disk and underwent groin lymphadenectomy in the transplanted limbs. The animals were followed up to the study's end point (Grade 3 rejection or > 150 days survival). Study design is presented in **Table 22**.

**Table 21. Study design.**

| Groups | # of animals | Immunosuppression regimen |
|---|---|---|
| Group 1 | 5 | No treatment |
| Group 2 | 6 | TAC administered intraperitoneally, at a dose of 1mg/kg/day |
| Group 3 | 6 | Single TAC-eluting disk subcutaneously implanted in the contralateral non-transplanted hind limbs |
| Group 4 | 7 | Single TAC-eluting disk subcutaneously implanted in the transplanted hind limbs |
| Group 5 | 4 | Single TAC-eluting disk subcutaneously implanted in the transplanted hind limbs with groin lymphadenectomy |

### 5.3.9 Orthotopic Hind-Limb Transplantation from Brown Norway to Lewis Rats

Experimental limbs from donor Brown Norway (BN) rats were transplanted to recipient Lewis (Lew) rats. In brief, donor and recipient animals were anesthetized with Nembutal (50mg/kg) or Ketamine (80mg/kg). Donor operations: The skin was incised proximal to the mid-thigh area. After exposing and cutting the femoral artery, vein, and nerve, the individual muscle groups and the femur were cut at the mid-shaft. The limb was flushed with heparinized Ringer's Lactate until clear fluid came from the vein. Recipient operation: After removing the similar portion of the leg, the donor leg was attached. Femoral bone osteosynthesis was achieved using an 18-gauge needle as an intramedullary rod. Femoral vessels were anastomosed using 10-0 Nylon interrupted sutures. The muscles were approximated using 5-0 vicryl and the skin were closed using 5-0 polyamide monofilament interrupted sutures [214].

### 5.3.10 Graft Survival Evaluation

Animals were monitored daily for clinical signs of rejection. Grade 0. No sign of rejection. Grade 1. Erythema. Grade 2. Progressive erythema and edema. Grade 3. Skin slough/epidermolysis. Grade 4. Necrosis as described previously [60, 214]. Grade 3 rejection or > 200 days survival was defined as the end point of the study.

### 5.3.11 Histology Evaluation

Skin and muscle samples were collected from the grafts at the end point, fixed in 10% neutral buffed formalin, embedded in Paraffin, sectioned at 3-µm thickness, and stained with hematoxylin and eosin (H&E) for microscopic examination in order to detect signs of rejection. Assessing histological rejection of skin and muscle was performed by pathologist blinded to the treatment groups and according to the established standard acute rejection grading scale as previously described [60, 214].

### 5.3.12 Evaluation of Donor Chimerism and Regulatory T cells in Recipients' Peripheral Blood, by Flow Cytometry

Cells were collected from lymph nodes, spleens, or peripheral blood of animals with long-term survival allografts >150 days post-transplant. Briefly, peripheral blood (300µl) was collected from the tail vein. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient density centrifugation method. For chimerism evaluation, mouse anti rat class I RT1Ac antibodies RTIAC-FIC was used for donor cell labeling (RT1n, BN rats). For Treg analysis, PBMCs were stained

with fluorochrome-conjugated mouse anti rat monoclonal antibodies for cell surface markers (CD11b/c, CD3, CD4, CD25, and CD45) or intracellular markers (FoxP3). These antibodies were added at the optimized concentrations stored in ice for 30 minutes, washed twice, and analyzed by flow cytometry using LSRII flow cytometer (BD Biosciences, San Jose, CA) using an open gate excluding small debris. PBMCs from naïve Lewis rats were used as negative control, and from Brown Norway rats were used as control for donor chimerism. Stained cells were run on LSRII-flow cytometer (BD Bioscience, San Diego, CA) and data were analyzed using then analyzed using FlowJO (Tree Star, Ashland, OR). Antibodies in various fluorochrome combinations were purchased from BD Biosciences (San Diego, CA) [220].

### 5.3.13 Evaluation of Donor-Specific T-cell Responsiveness using Mixed Lymphocyte Reaction (MLR) Assay

MLR was performed to evaluate donor-specific T-cells responsiveness in animals with DTx and/or DnonTx at post-operative days 50. PBMCs were isolated from the peripheral blood of the recipients (n=3) by gradient density centrifugation and used as the responder cells and labelled with carboxyfluorescein succinimidyl ester (CFSE) dye. Responder cells (2 x 105/well) were cultured with irradiated (3000 rad) naïve BN splenocytes as the stimulator cells (1 x 105/well) in triplicates in round-bottom 96-well plates. All assays included appropriate negative and positive control cultures (unstimulated and stimulated naïve Lewis PBMCs). MLR were cultured in complete RPMI medium (RPMI +10% FBS+1% L-glutanmine) and incubated at 37°C in 5% CO2 atmosphere for 5 days. After which cells were collected from the plate and stained with T cell surface markers (CD3, CD4) for proliferative studies measured by flow cytometry (CFSE analyses of specified gated CD3+ CD4+ T cells). The reactivity of the T cells to the donor cells was assessed

by relative response as follow: RR% = (Recipient response – unstimulated negative response) / stimulated positive response x 100.

### 5.3.14 Evaluation of Immunosuppression Related Toxicity:

Kidney and liver toxicity markers in blood including serum creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were analyzed by standard clinical chemistry methods in the Central Laboratory of University of Pittsburgh Medical Center (UPMC). Results were compared to naïve age-matched Lewis rats. Kidney function was evaluated by measuring creatinine clearance (CrCL) on the day before surgery (Day -1), and on the day 120 after surgery. Twenty four-hour urine collections were performed to measure urinary creatinine levels (Cr urine), and blood samples were collected for serum creatinine levels (Cr serum). Creatinine levels were measured using a Beckman autoanalyzer employing a modification of the Jaffe procedure (Beckman Instruments, Fullerton, CA, USA). CrCl, (ml/min) were calculated (Cr urine x Urine volume)/Cr serum)/1440) and compared [254]. Fasting blood glucose concentration was measured in the venous blood of the tail vein using Accu-chek® sensor regularly on days 0, 30, 60, and 120 post-transplantation. Intraperitoneal glucose tolerance test (IPGTT) was performed on day 125 for the animals with long-term survival allografts in group 2 and 4. Animals were fasted for 12 hours, glucose dose (2g/kg of body weight) was administered intraperitoneally. Blood glucose concentration was measured in the venous blood of the tail vein using Accu-chek® sensor on 0, 30, 60. 90, and 120 min after the glucose injection [217]. We also monitored the animal feeding behavior and the change in the body weight.

### 5.3.15 Quantification of TAC Concentration in Tissues:

The skin sites for tissue sampling were wiped down three times with ethanol-soaked gauze to remove residual ointment on the surface. Skin and muscle were frozen with liquid nitrogen and pulverized in pestle and mortor to fragment the frozen tissues samples into fine pieces. Tissues were weighted and homogenized with cold methanol (1ml) in homogenization tubes using Mini-BeadBeater-1 (Cole-Parmer North America) for cell disruption. The homogenate was sonicated for 1 hour at 25 °C and then kept overnight at 4°C to allow for the complete extraction of the drug from the tissues. The homogenate was transferred to an appropriately labeled micro centrifuge tube and centrifuged at 2100 ± 100 rpm for 10 min. The supernatant was transferred to a labeled glass vial and evaporated by sample concentrator and the drug residue was reconstituted with blood (1ml). Tissue drug concentrations are expressed as ng/g of tissue weight. Extraction recovery of TAC from skin and muscle were 87% and 89%. To control for residual ointment on the skin, ointment was applied on limbs (n=4) and immediately cleaned with ethanol-soaked gauze. Biopsies from skin were collected and analyzed for TAC concentration. The highest TAC concentrations from residual ointment that remained on the skin after wiping off were minimal (19±9ng/g) compared to the actual tissue concentrations.

### 5.3.16 Quantification of TAC in Blood by Liquid chromatography–mass spectrometry (LC-MS/MS):

Fifty microliters of blood containing an unknown concentration of tacrolimus was added to a conical centrifugation tube, followed by two hundred microliters of a solution of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) to precipitate blood proteins. Five hundred microliter of acetonitrile

containing an internal standard (ascomycin) at a concentration of 15ng/ml was then added and the mixture was vortexed at 3000 rpm for 2 minutes. Samples were centrifuged at 13,000 rpm for 3 minutes with the supernatant being poured off and collected into LCMS vials for analysis. Analysis was performed using a validated, reverse phased method for the detection of TAC in blood on a Waters micromass Quattro micro API mass spectrometer operated in a positive electrospray ionization mode, utilizing multiple reaction monitoring, after injection of 20 µL of sample. The Waters 2795 Alliance Separations Module was equipped with a nova-pack® C18 column, 2.1 x 10 mm cartridge (Waters # 186003523) heated to 55° C. Analytes were effectively separated using a gradient elution consisting of an aqueous mobile phase (95% $H_2O$ / 5% MeOH) and an organic mobile phase (100% MeOH), at a flow rate of 0.6 mL per minute. Mobile phases also contained 0.1% formic acid ($CH_2O_2$) and 2mM ammonium acetate. Monitored parent to product mass transitions for TAC and ascomycin were 821.63 → 768.33 and 809 → 756 m/z, respectively. TAC had a retention time of 1.2 minutes. The standard curve was linear for concentrations ranging from the lower limit of quantification (LLQ) value of 2 ng/ml up to concentration values as high as 40 ng/mL with an $R_2$ value of 0.9996 (With the lower limit for $R_2$ acceptability being defined as 0.99). Limit of detection (LOD) was 0.1 ng/ml. Both intra- and inter-day precision were acceptable (C.V. <10%, n=3) at concentrations of 4.3, 15.7, and 24.6 ng/mL [140].

### 5.3.17 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 statistical software for windows (GraphPad Software, La Jolla, CA, USA). Six animals for each group was selected to achieve adequate power with alpha = 0.05 and beta (power) = 0.8 considering 30% variability, based on previous experience and published results, and looking for 40% difference in the effect of site-

specific immunosuppression. Graft survivals in each group were plotted using the Kaplan-Meier method, and the differences in the median allograft survival between groups was analyzed using the log-rank test. All data sets were checked for normality. Data sets were expressed as means ± standard deviation, and Student t test and/or analysis of variance (ANOVA) test was used to compare the difference between these groups. Post hoc test was used to do multiple comparisons. A p value $< 0.05$ was considered as statistically significant difference. Statistically significant data were presented as follows: *P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. Statistical tests are specifically indicated under each figure. Data are expressed as mean ± SD.

## 5.4 Results

### 5.4.1 Morphology, Drug Loading, Encapsulation Efficiency, and In-Vitro Degradation of TAC-PCL Disk

The prepared disks have a smooth surface with a 1 cm diameter and 0.5cm thickness. The pores diameter ranged from 10 to 20 µm as measured from SEM images as shown in **Figure 40 (a, b)**. The experimental results showed that our method could encapsulate 86.7±6.3% (w/w) TAC in the disk with a drug loading of 4.3±0.3%. To determine if TAC loading changes PCL matrix degradation, we analyzed the percent change in mass of unloaded PCL and 5 mg TAC loaded PCL matrices over four weeks. Changes in mass were similar for the two matrices (un-loaded and loaded). At the first week, mass was largely unchanged. However, between days 14 and 30, the mass decreased similarly in the two matrices. 5.5 ± 1% mass reduction in unloaded PCL and 6.3 ± 0.7 % mass reduction in PCL-TAC. Matrices remained intact after 12 weeks.

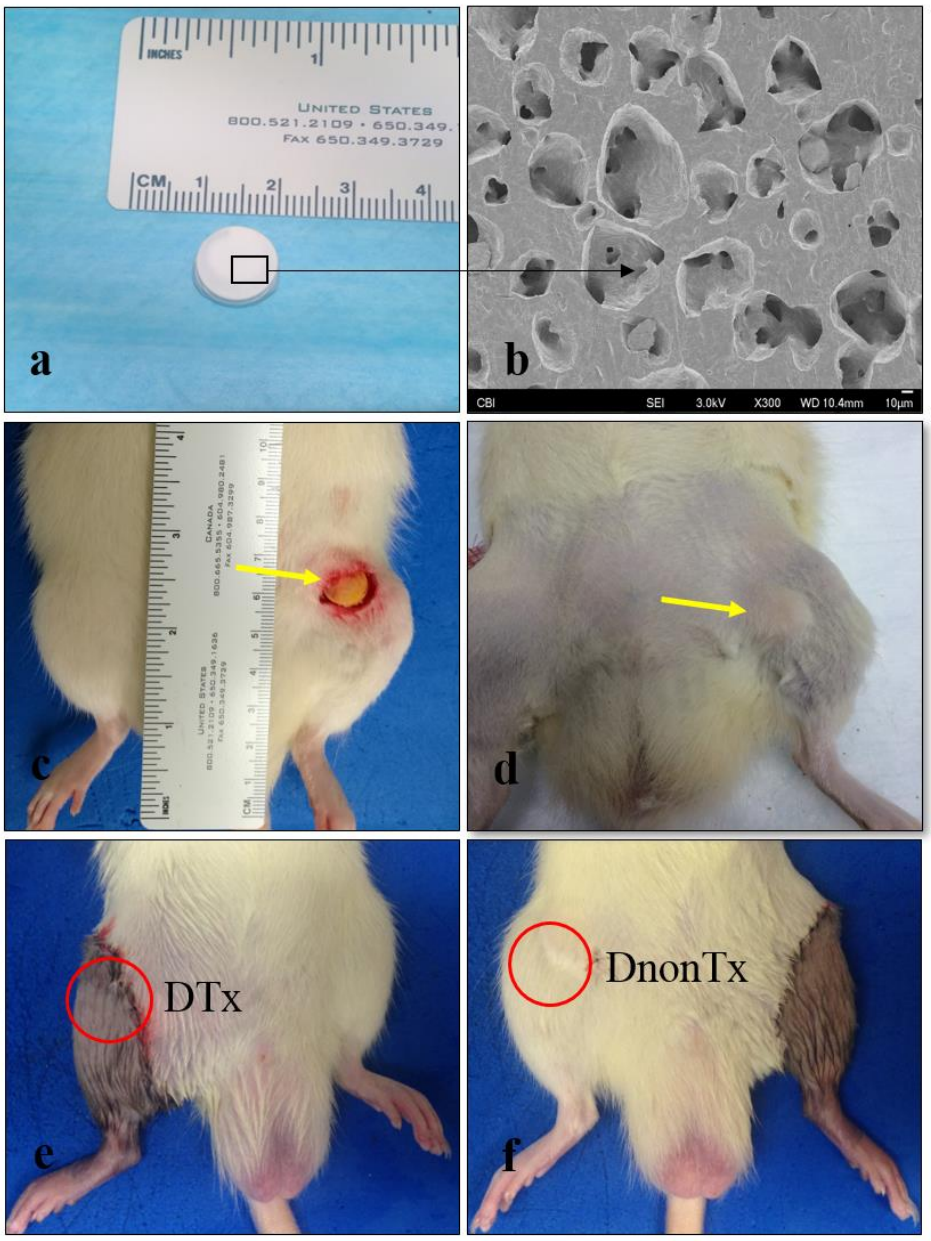



**Figure 40. Morphology of TAC loaded PCL disk. Macroscopic appearance of TAC loaded PCL disk (5mg, 5%w/w) with 1 cm in diameter and 0.5cm thick (a). Scanning electron microscopic images of TAC loaded PCL disk show the microstuture and diameter of the pores at magnification x300 (b). Subcutaneous implementation of TAC-PCL disk at the hind limb of the rat (c and d). Subcutaneous implementation of TAC disk in the transplanted limb DTx (e) or in the contralateral non-transplanted limb DnonTx (f). The Red circle marks the disk location.**

### 5.4.2 In-Vitro and In-Vivo Release of TAC from PCL Disk

The in-vitro release profile of TAC from PCL disks is shown in **Figure 41**. TAC was released in a gradual and sustained manner with a total release of 37.7±4.4 % over 30 days. Initial burst release was observed in the first few days, then the cumulative released amount was linear with time t (zero-order release kinetics). Five milligram of TAC loaded PCL disk released 17833±2983ng/ml (22180±3711nM) within 24-hour time point, and 34333±4041ng/ml (42703±5026 nM) within 30 days. Single TAC-eluting disk (5mg, 5 % w/w) in the hind limbs of Lew rats as shown in **Figure 40 (c, d)** resulted in relatively rapid initial burst release on post-operative day 1 reaching 17 ± 8 ng/ml at 12 hour and 14.9±2 ng/ml at 24 hours. After this, the concentrations dropped to 8.9 ± 3.4 ng/ml by postoperative day 7 and to 5.3 ± 1.9 ng/ml by postoperative day 14. After this, the concentrations were low but stable between 2 to 5 ng/ml for nearly 100 days. Then, the concentrations dropped to less than 2 ng/ml. Average TAC concentrations in the blood over time is shown in **Figure 42 (a)**. Significantly higher concentrations were detected in the skin, muscle, sciatic nerve, and DLNs collected from the disk implanted limbs compared with the contralateral limbs (***p=0.0002, ***p=0.0004, ****p<0.0001, ***p<0.0002). Average TAC concentrations in the skin, muscle, sciatic nerve, and DLNs collected from both the disk implanted limbs and the contralateral limbs are shown in **Figure 42 (b).**

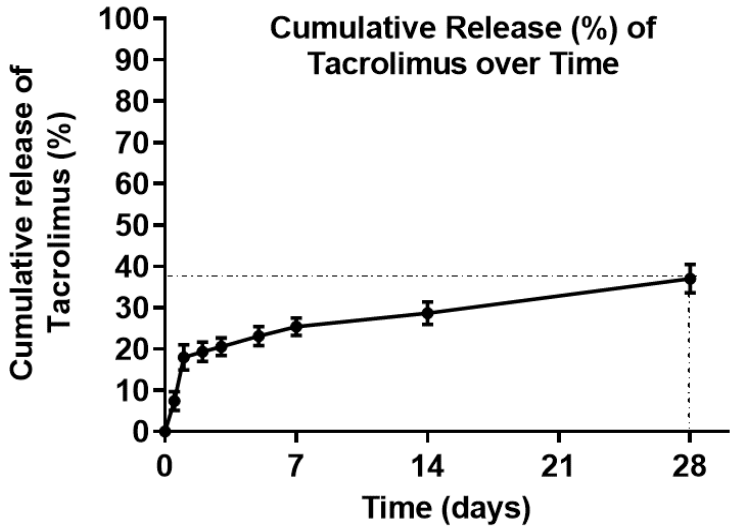



**Figure 41. Plot of the cumulative release (%) of TAC in-vitro in phosphate buffered solution (PH=7.4) at 37c0 vs. Time (Mean ± SD, n=4).**

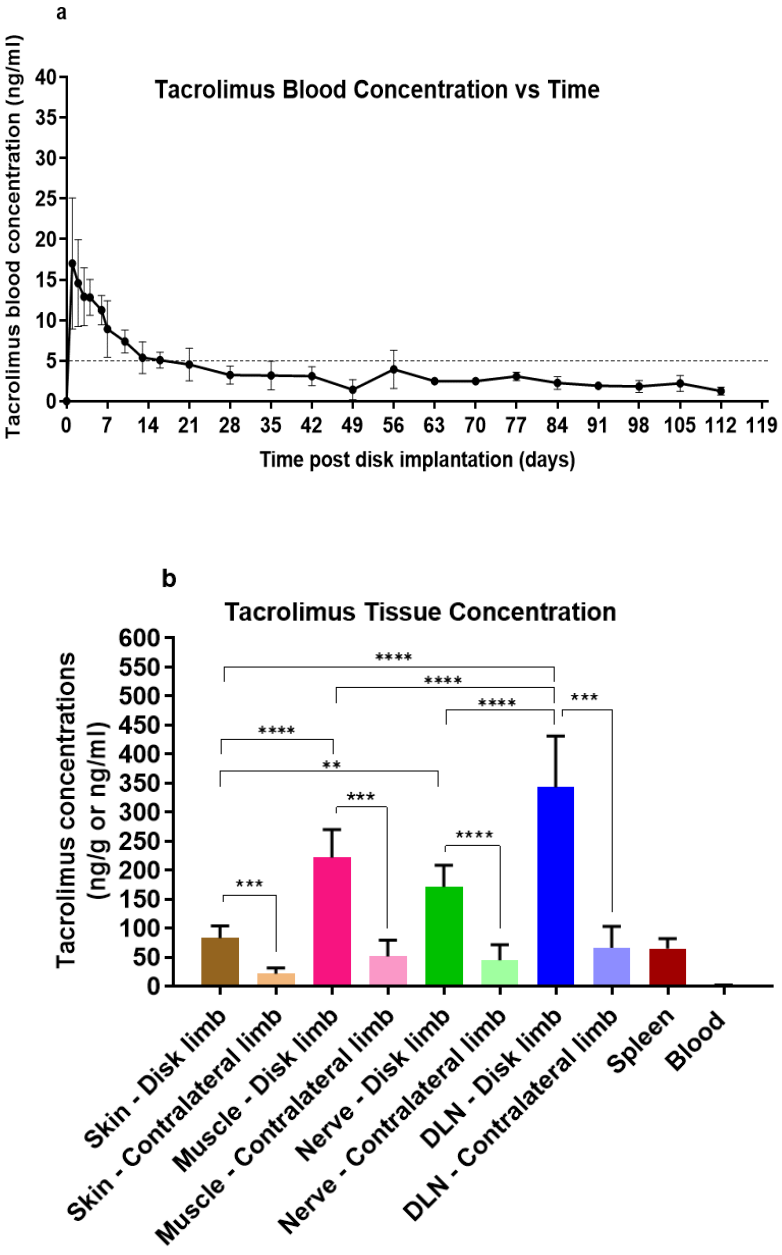



**Figure 42. Tacrolimus release from the disk into the blood and tissues following subcutaneous implantation of TAC disk in the hind limbs of rats. (a) Tacrolimus concentration in blood was measured by LC-MS/MS in the blood (ng/ml) over time. Data presented as mean+SD, n=6/group. (b) Tacrolimus concentration in tissues was measured by LC-MS/MS in the tissues (ng/g) collected from the disk implanted limbs and from the contralateral limbs at 120 days. Significantly higher concentrations were detected in the skin, muscle, nerve, and DLNs collected from the disk implanted limbs compared with the contralateral limbs (***p<0.001,**

*****p<0.001, ****p<0.0001, ***p<0.001). Data presented as mean+SD, n=6/group. Paired t test is used for comparisons within a group between the two sites of biopsy collections and one-way ANOVA with Tukey's multiple comparisons test was used for comparisons between different tissue groups. Significant differences are indicated as * p < 0.05, ** p < 0.01, and ***p < 0.001.**

### 5.4.3 Single TAC-Eluting Disk within the Allograft Enables Long-Term Survival of Orthotopic Rat Limb Vascularized Composite Allografts (>200 days) via Sustained Site-specific Immunosuppression

To test the hypothesis that sustained site-specific immunosuppression at the allograft using a single TAC-eluting disk within allografts may prevent VCA rejection, orthotopic rat hind limb allo-transplantations were performed from Brown Norway-to-Lewis rats. A single TAC disk (5mg, 5 % w/w) was subcutaneously implanted in the transplanted hind limbs (DTx) or in the contralateral non-transplanted hind limbs (DnonTx) (**Figure 40 (e and f))**. Kaplan-Meier survival plot of time to reach Grade 3 rejection or >200 days survival of transplanted limbs is demonstrated graphically in **Figure 43**. Macroscopic appearance of the transplanted hind limbs at the end point is demonstrated in **Figure 44.** Transplanted animals in Group 1 (n=5) did not receive any treatment. The median survival time to grade 3 rejection was 8 days. Transplanted animals in Group 2 (n =6) that treated with daily intraperitoneal injection of 1-mg/kg tacrolimus (STAC) throughout the study duration, sustained their allografts for >150 days. Transplanted animals in Group 3 (n=6) that received DnonTx, rejected their allografts. The median time to grade 3 rejection was 70 days. This indicates that the TAC disk was effective in prolonging the allograft survival when it implanted at the transplant site, but not when implanted remotely. Locally delivered TAC

at the transplant site was effective in preventing rejection by inhibiting the local alloimmune response at the allograft and DLNs (alloantigen/T cells interactions). Single TAC disk implanted in the transplanted hind limb (DTx) (Group 4, n=6/7) and/or single TAC disk implanted in the transplanted hind limb with lymphadenectomy (Group 5, n=4) significantly increased the allograft survival (>150 days) compared to other groups. However, animals in Group 5 eventually rejected their allografts. The median survival time to grade 3 rejection was 175 days. This indicates that DLNs are important sites where alloantigen is presented to T cells, and that under the influence of immunosuppression the ongoing local alloimmune response can be inhibited and the allograft survival can be maintained. Removal of DLNs results in dendritic cell/T-cell interactions occurring at other remote secondary lymphoid tissue, and subsequently induction of rejection in the absence of TAC at these sites. Group 4 vs. group 1 (***p=0.0003), group 4 vs. group 2 (p>0.05), group 4 vs. group 3 (***p=0.0002), group 4 vs. group 5 (p=0.064). Group 5 vs. group 1 (**p=0.0049), group 5 vs. group 2 (p=0.19), group 5 vs. group 3 (**p=0.0038). P values were calculated by log-rank test.

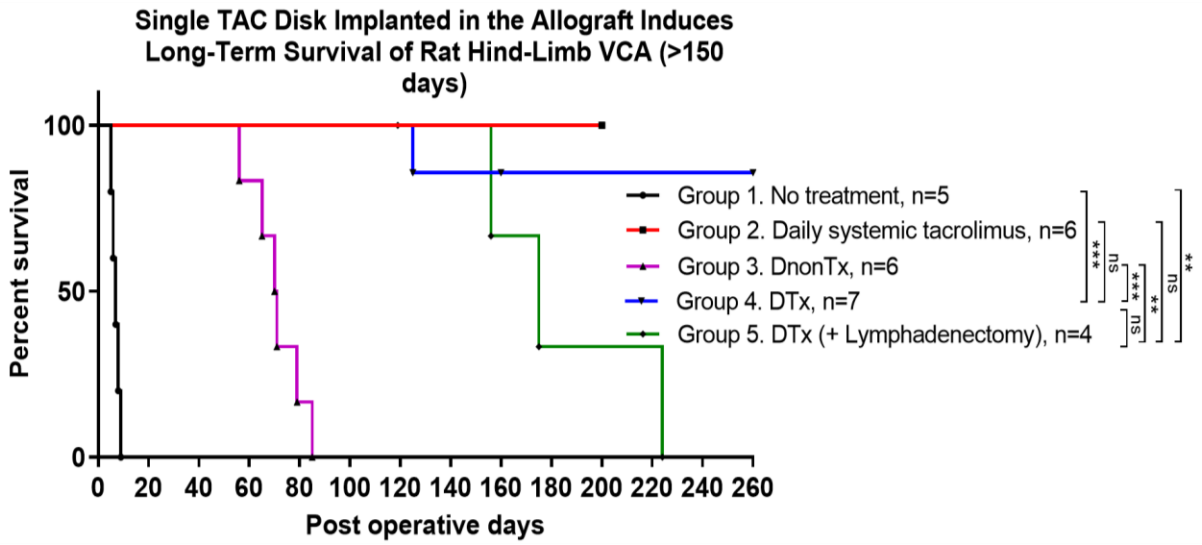



**Figure 43. Single TAC disk implanted in the allografts induces long-term survival of rat hind-limb VCA (>150 days). Kaplan-Meier graft survival curves for the allograft of Brown Norway-to- Lewis orthotopic hind limb transplantation of the five groups. Group 1 (n =5) was left untreated. Group 2 (n =6) was treated with daily intraperitoneal injection of 1-mg/kg tacrolimus (STAC). Group 3 (n=6) was received TAC loaded PCL disks subcutaneously implanted in the contralateral naive hind limbs (DnonTx). Group 4 (n=7) was received TAC loaded PCL disks subcutaneously implanted in the transplanted hind limbs (DTx). Group 5 (n=4) was received DTx with lymphadenectomy. Group 4 vs. group 1 (***p=0.0003), group 4 vs. group 2 (p>0.05), group 4 vs. group 3 (***p=0.0002), group 4 vs. group 5 (p=0.064). Group 5 vs. group 1 (**p=0.0049), group 5 vs. group 2 (p=0.19), group 5 vs. group 3 (**p=0.0038). P values were calculated by log-rank (Mantel-Cox) test.**

### 5.4.4 Histological Evaluation of the Long-Term Surviving Allografts of Animals with DTx Confirmed the Absence of Histopathologically Evident Rejection

To confirm the clinical evaluation of the allografts in all groups at the study's end point, skin and muscle biopsies were collected for histopathological evaluation as shown in **Figure 44**. Macroscopic status of allografts from animals which received DnonTx (Group 3) showed typical signs of clinical rejection by day 70. Histology of samples from these allografts showed signs of rejection. Skin revealed epidermal loss with intense and diffuse dermal inflammatory infiltrate composed of mononuclear cells (Grade 3), and muscle revealed diffuse, dense inflammatory infiltrates, myocyte necrosis, fibrotic replacement of myocytes, and edema (Grade 2). Macroscopic status of allografts from animals received DTx (Groups 4) didn't show signs of clinical rejection (> 200 days). Skin and muscle revealed normal and intact tissue structure without lymphatic infiltrate, edema, or necrosis. Allografts from animals in Group 5 (DTx and lymphadenectomy) showed sign of clinical rejection by day 175 which was confirmed by the histological evaluation. Skin revealed, and muscle revealed.

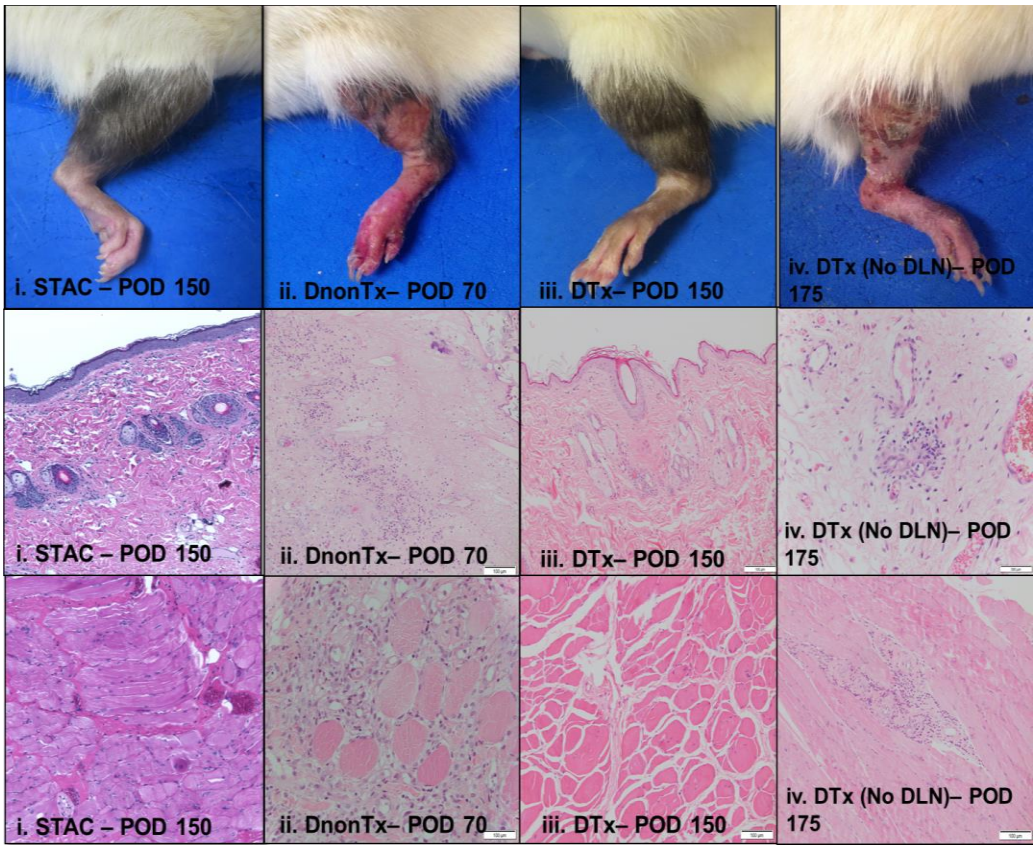



**Figure 44. Clinical and histopathological evaluation of hind limb transplants. Representative macroscopic images of hind limb allografts at the end point (Garde 3 rejection or >200 days survival). (i) Group 2 (STAC), day 150 post-transplant. Healthy appearance of the transplanted limb with normal hair and nail growth. (ii) Group 3 (DnonTx), day 70 post-transplant. Note the signs of clinical rejection (Grade 3), erythema with some edema at the foot, epidermolysis and sloughing of epidermal tissue including hair. (iii) Group 4 (DTx), 150-day post-transplant. Healthy appearance of the transplanted limb with normal hair and nail growth. (iv) Group 5 (DTx+lymphadenectomy). Note the signs of clinical rejection (Grade 3), erythema, epidermolysis and sloughing of epidermal tissue including hair. Representative photomicrographs of the histology (H&E staining) of skin (Middle panel, i-iv) and gastrocnemic muscle (Lower panel, i-iv) from experimental groups. Rejected grafts (ii and iv) showed severe cellular infiltrations, edema formation, and necrosis while non-rejected grafts (i and iii) showed normal and intact tissue structure with healthy basal keratinocyte layer. Magnification, 20x; scale bars, 100 µm.**

### 5.4.5 Single TAC Disk Subcutaneously Implanted in the Hind Limbs provides Low Systemic and High Loco-Regional Drug Concentrations

The average concentrations of TAC in the blood of transplanted animals that received TAC-PCL disk therapy (Group 3, 4, and 5) post-transplant are shown in **Figure 45 (a)**. Average blood concentrations of TAC on post-operative day 7 was 9.6 ± 3.5, 10.5 ± 5, and 7.4±1 ng/ml in group 3, 4, and 5, respectively ($p>0.05$). Initial burst release of TAC was observed on post-operative day 2 reaching 18.3 ± 6.6, 15 ± 7.6 and 13.5 ± 7ng/ml in groups 3, 4, and 5, respectively ($p>0.05$). After this, the concentrations dropped to 9.9 ± 5.3, 8.5 ± 2.1, and 5.2 ± 1 ng/ml by postoperative day 14, respectively ($p>0.05$). Subsequently, the concentrations stayed low but stable between 2 and 5 ng/ml until the end point for group 3, and until postoperative day 105 for group 4 and 5. After this, the concentrations dropped to less than 2 ng/ml. Animals in Group 3 rejected their allografts by day 70. However, animals in Groups 4 and 5 maintained their allografts despite sub-therapeutic blood levels of TAC. Animals in Group 5 eventually rejected their allografts by day 175. The average trough blood concentrations of TAC were between 5-15 ng/ml in animals received daily systemic TAC (Group 2). The average weekly levels of TAC in the blood of transplanted animals that received TAC-PCL disk therapy was significantly lower than the values observed in the animals that received systemic TAC therapy (****$p < 0.0001$) as shown in **Figure 45 (b)**.

We evaluated the local drug exposure in all groups by measuring TAC concentration in the tissues collected from the disk implanted limbs and the contralateral non-transplanted limbs post-transplant at the study end point. Data are shown in **Figure 46 (a and b)**. In groups 3 and 4, the average concentrations of TAC in the tissues (DLN, sciatic nerve, skin, and muscle) collected from the disks implanted limbs (non-transplanted limbs for group 3, and transplanted limbs for group

4) at study end point were significantly higher than the concentrations of TAC in the tissues collected from the contralateral limbs (transplanted limbs for group 3, and non-transplanted limbs for group 4) ($p<0.05$). Single TAC-eluting disk implanted in the transplanted limbs (group 4) results in significantly higher locoregional concentrations of TAC in the allograft tissues such as DLN, muscle, and skin, when compared to the concentrations observed in the blood (220, 123, and 34-fold higher). Single TAC-eluting disk implanted in the contralateral non-transplanted limbs (group 3) results in significantly higher locoregional concentrations of TAC at the contralateral non-transplanted limb tissues such as DLN, muscle, and skin, when compared to the concentrations observed in the blood (163, 87 and 29-fold higher). The average concentrations of TAC in DLN, muscle, skin collected from the disks implanted limbs (transplanted limbs for group 4) were significantly higher than the concentrations in the tissues collected from animals received systemic TAC therapy (transplanted limbs for group 2) at study end point (****$p<0.0001$, ****$p<0.0001$, **$p=0.002$).

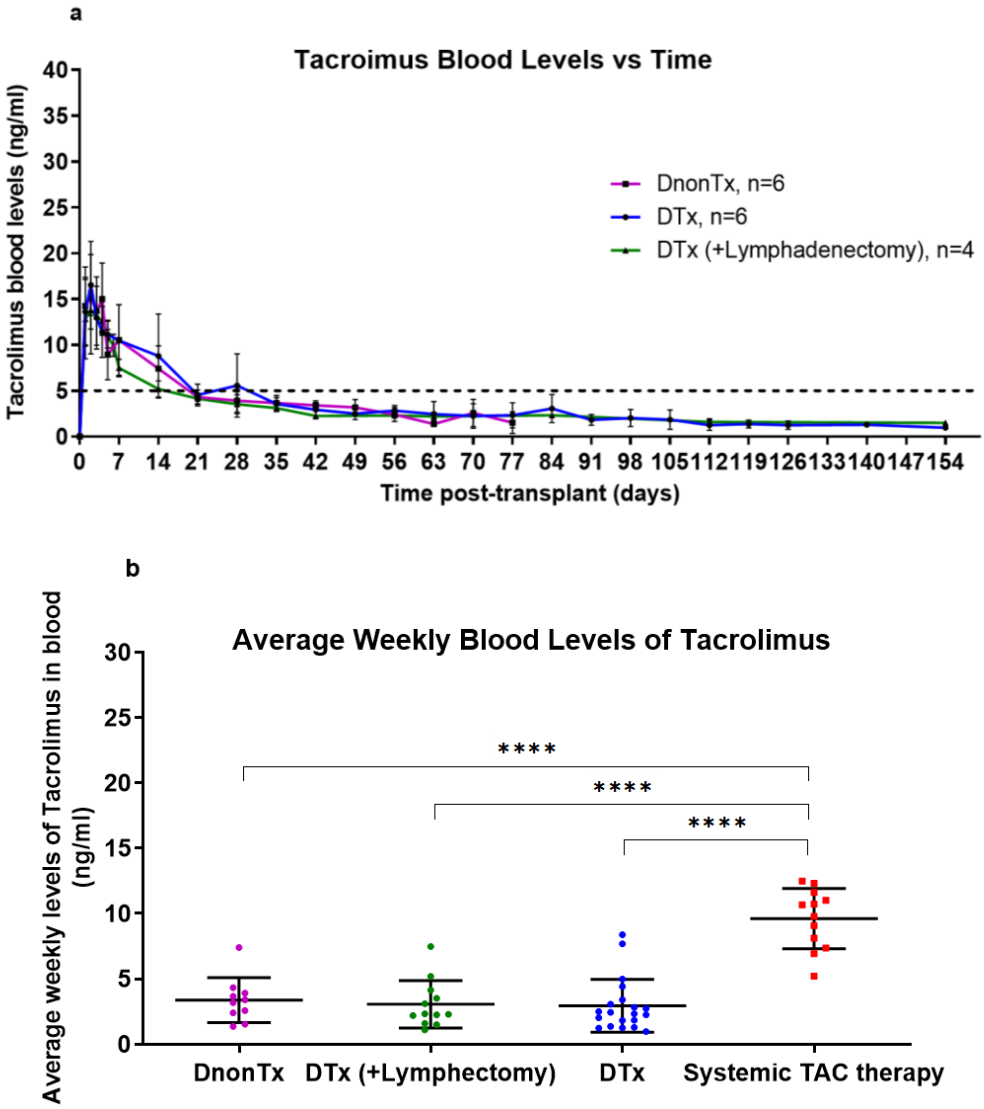



**Figure 45. Concentrations of tacrolimus in the blood are lower with TAC-PCL disk therapy. (a). Concentrations (ng/ml) of tacrolimus in the blood of transplanted animals received one TAC disk either in the contralateral non-transplanted limb DnonTx (Group 3, n=6) or in the transplanted limb DTx (Group 4, n=7 and Group 5, n=4) over time. Data are presented as mean ± SD. (b). Average weekly concentrations of tacrolimus in the blood of transplanted animals received one TAC disk (Groups 3, 4, 5) and/or systemic TAC therapy. Each data point represents the mean value of tacrolimus measurements collected weekly exactly at the seventh day of each week (starting from postoperative week 1 etc) until the end of the study. Statistical analyses of the differences between the groups are shown. Data are presented as mean ± SD, ****P<0.0001 by ANOVA.**

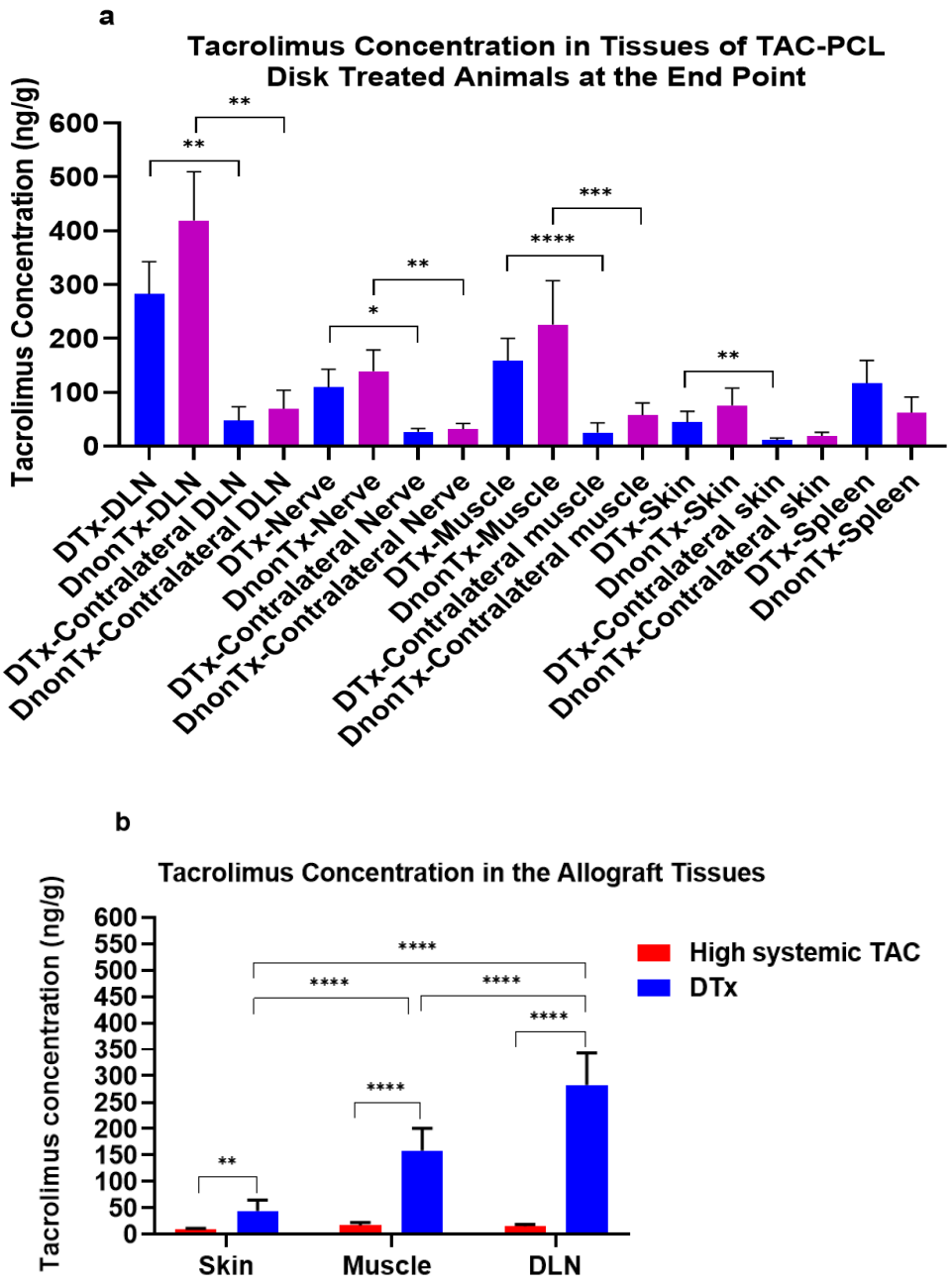



**Figure 46. Average tacrolimus concentrations in allograft tissues are higher with TAC-PCL disk therapy. (a). Average tacrolimus concentrations (ng/g) were measured by LC-MS/MS in the allograft of TAC-PCL disk therapy treated animals. DnonTx, one TAC disk in the contralateral non-transplanted hind limb (Group 3); DTx, one TAC disk in the transplanted hind limb (Group 4) at the end point. DLN, draining lymph node (lymph nodes around the implanted TAC disk); Disk muscle, muscle around the implanted TAC disk; Disk skin, skin around the implanted TAC disk. Disk nerve, nerve around the implanted TAC disk. (b). Average tacrolimus**

**concentrations in the transplanted hind limb tissues (DLNs, skin, and muscle) of TAC-PCL disk treated or systemic tacrolimus treated animals (>100 days). Each data point represents the mean value of tacrolimus measurements across the experimental group at the study end point. Statistical analyses of the differences between the tissue groups or 2 sites of biopsy collections are shown. Data are presented as mean ± SD. Paired t test is used for comparisons within a group between the two sites of biopsy collections, and independent t test is used for comparisons between two groups, and one-way ANOVA with Tukey's multiple comparisons test was used for comparisons between different tissue groups. Significant differences are indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.**

### 5.4.6 Levels of Hematopoietic Chimerism and Systemic T Regulatory Cells in Peripheral Blood Appeared Unaffected with TAC-Eluting Disk within Allograft.

The percent of donor cells among PBMCs and CD4+CD25+FoxP3+ cells in the peripheral blood was evaluated in the animals received DTx (>150 days), systemic TAC, and naïve age-matched Lew and shown in **Figure 47 (a-c)**. No significant difference was observed in the percent of donor cells among PBMCs of animals received DTx (>150 days) (n=3) as compared to animals received systemic TAC (n=4) and control naïve (age-matched) Lew (n=4) (0.6±0.7 vs. 0.4±0.2 and 0.27±0.05, P>0.05, respectively). Despite that the percent of CD4+CD25+FoxP3+ cells in the peripheral blood of animals received DTx (>150 days) (n=6) was relatively higher as compared to the values observed in the animals received systemic TAC (n=3), and naïve (age-matched) Lew (n=6), the difference was not significant (4.3±1 vs. 1.9±1.6 and 4±2, P>0.05, respectively).

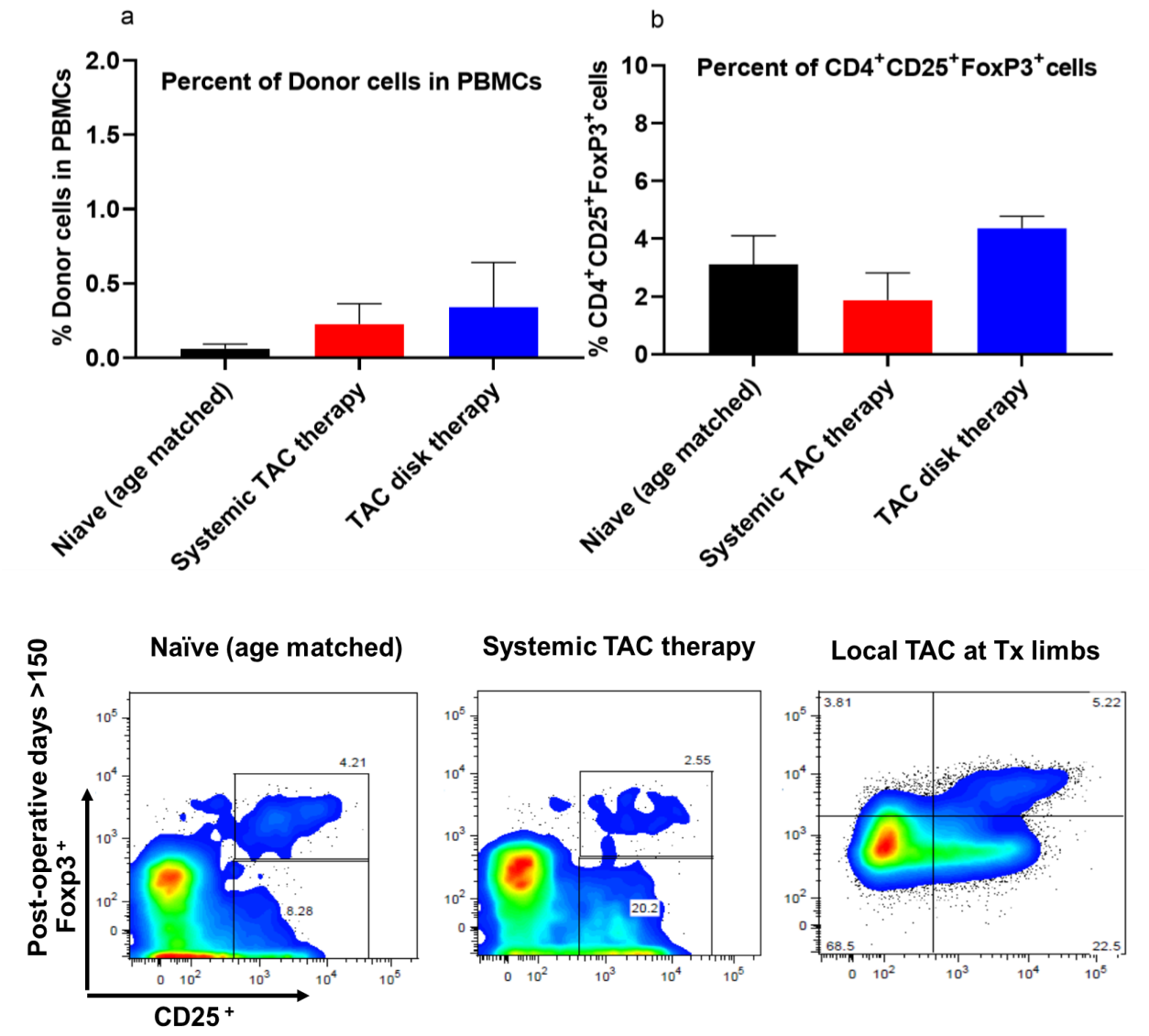



**Figure 47. Level of hematopoietic chimerism (a), and percent of CD4+CD25+Foxp3+ (b) in the peripheral blood of animals with long-term survival allografts in group 2 and 4 (>150 days). Naïve animals were used as negative controls. Representative FACS images for peripheral blood of CD4+CD25+Foxp3+ from control naive age-matched animals and animals in group 2 and group 4 is presented (c). Numbers in dot plots indicate percentages of positive cells (Foxp3+ cells within CD4+CD25+Tcells) in that quadrant. FACS indicates fluorescence-activated cell sorter. Statistical analyses between the groups are shown. Data shown as mean+SD. P values were calculated by one-way ANOVA with Tukey's multiple comparisons test.**

### 5.4.7 Systemic Lymphocyte Proliferative Response Appeared Unaffected with Locally Delivered TAC

To confirm whether locally delivered TAC is capable of locally inhibiting alloimmune response and preventing the allograft rejection by site-specific immunosuppression, we assessed the systemic lymphocyte proliferative response to the donor antigen in the animals received TAC disk in Tx limbs (Group 4) and non-Tx limbs (Group 3) at specific time points post-transplantation. As shown in **Figure 48**, PBMCs of animals that received TAC disk in the transplanted limbs or non-transplanted limbs (POD50) showed normal or slightly activated lymphocyte proliferative response to donor PBMCs as compared to control naïve animals (84.5±7.8% and 98.8±15% vs. 94±3.4%, $p>0.05$). High proliferation was observed when naïve LEW PBMCs were stimulated with donor (BN) PBMCs as positive control. No proliferation was observed when the Lewis PBMCs were cultured alone and/or not labeled with CFSE as negative controls.

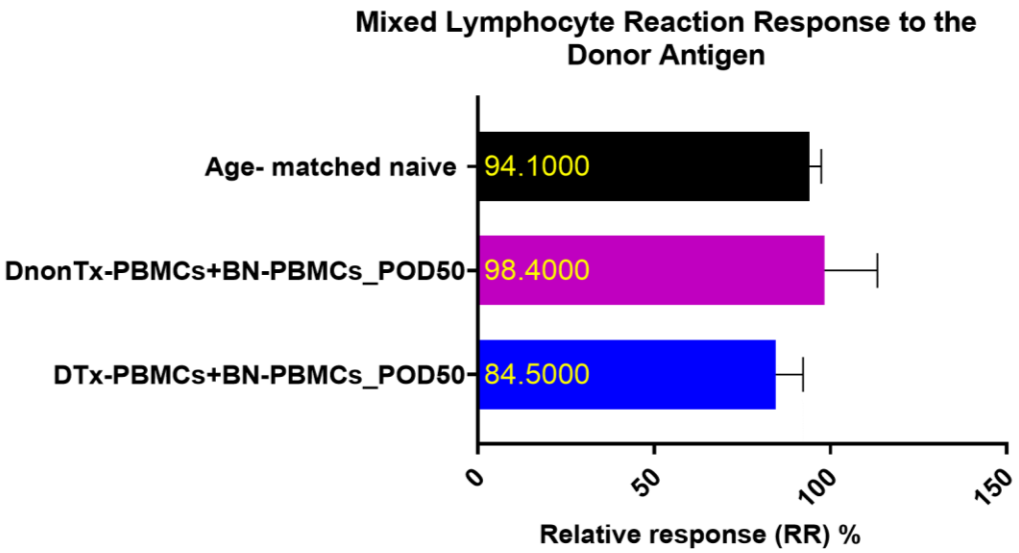



**Figure 48. Mixed lymphocyte reaction response to the donor splenocytes. Relative T cell proliferative response of animals with TAC disks in the contralateral non-transplanted limbs DnonTx (n=3) or in the transplanted limbs DTx (n=3) on POD 50 to the donor splenocytes. Statistical analyses between the groups are shown. Data shown as mean+SD. P values were calculated by one-way ANOVA with Tukey's multiple comparisons test.**

### 5.4.8 Single TAC-Eluting Disk within Allograft does not Result in Renal and Liver Toxicity

To test whether a single disk loaded with 5 mg of TAC could induce nephrotoxicity, the kidney function as measured by the BUN levels and the creatinine clearance was evaluated in TAC disk group (n= 6), naïve (age matched) group (n=6), syngeneic Tx (age matched) group (n=4), and systemic TAC group (n= 4) at two-time points, pre-transplant (day -1) and post-transplant (day 120) during the study period as shown in **Figure 49**. No significant change was observed in the average CrCl in the naïve (age matched) group, syngeneic Tx (age matched) group, and TAC disk group at the two-time points, pre-transplant (day -1) and post-transplant (day 120) ($p=0.87$, $p=0.32$, $p=0.14$). The greatest deterioration in CrCl was observed in group 2 where the transplanted animals received daily systemic TAC therapy (1mg/kg/day) with 1.8±0.2 ml/min at day 120 post-transplant as compared to the average CrCL pre-transplant (**$p=0.0036$), and as compared to the average CrCL at day 120 post-transplant in the other groups (**$p<0.01$). Systemic TAC therapy vs. TAC disk therapy group (**$p=0.008$), Systemic TAC therapy group vs. Syngeneic Tx (age matched) (*$p=0.01$), systemic TAC therapy group vs. Naive (age matched) (**$p=0.0031$). There was no significant difference in BUN levels between TAC disk group and naïve (age matched) animals ($p=0.2$). BUN levels were significantly higher in the animals received systemic TAC therapy as compared to levels observed in Naive (age matched) animals (**$p=0.0106$).

To test whether a single disk loaded with 5 mg of TAC could induce hepatotoxicity, the liver function as measured by ALT and AST levels was evaluated in TAC disk group (n= 3), systemic TAC group (n= 3), and naïve (age matched) group (n=3) at POD 120 as shown in **Figure 50**. Animals from TAC disk group did not show significant difference in the ALT and AST levels

as compared to the naïve (age matched) animals (p=0.098 and p=0.23), demonstrating a stable liver function. While animals received daily systemic TAC therapy (1mg/kg/day) showed increase in all the biochemical parameters. Significantly higher AST levels in systemic TAC therapy vs. Naive (age matched) (***p=0.0002) and vs. TAC disk therapy group (****p<0.0001).

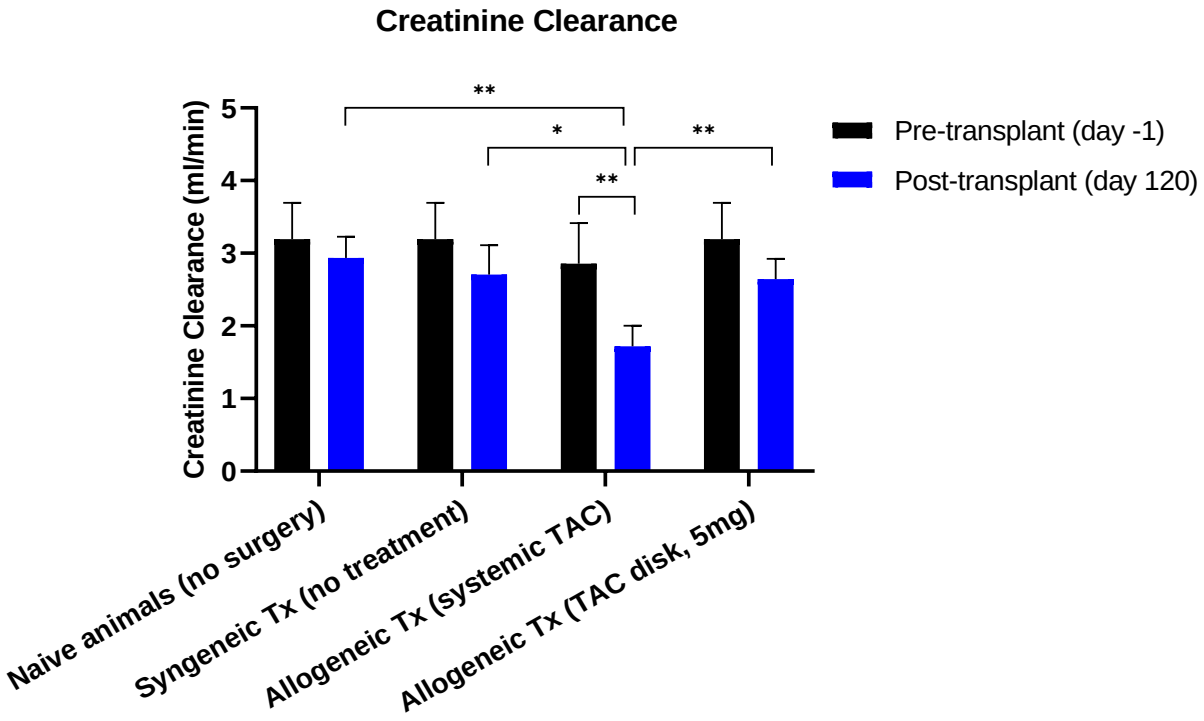
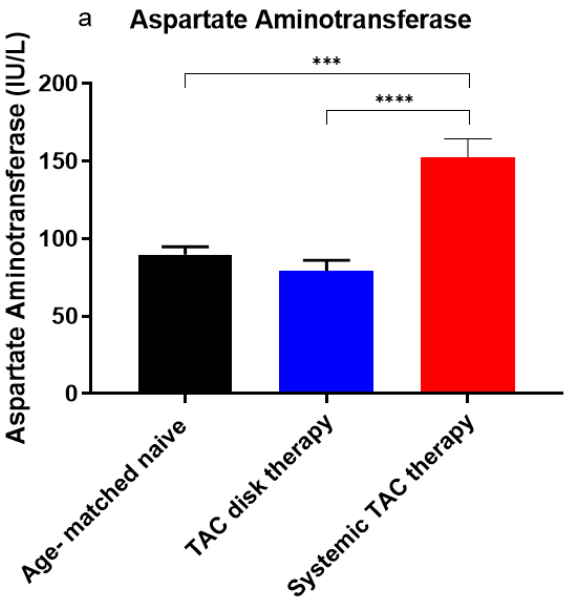



**Figure 49. TAC disk therapy did not induce renal toxicity. Measurements of the creatinine clearance in the naïve age matched group (n=6), syngeneic Tx group (n=4), systemic TAC group (n= 4), and TAC disk group (n= 6) at two-time points, pre-transplant (day -1) and post-transplant (day 120) during the study period. Statistical analyses of the differences between the groups are shown. Data shown as mean+SD. Intra-group difference was evaluated by paired t test. Inter-groups differences were evaluated by one-way ANOVA with Dunnett's multiple comparisons test. Significant differences are indicated by *p < 0.05, **p < 0.01.**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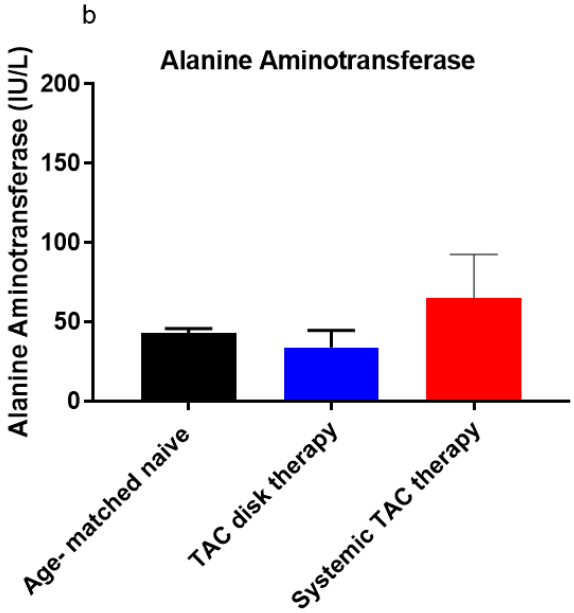
TAC disk therapy
Systemic TAC therapy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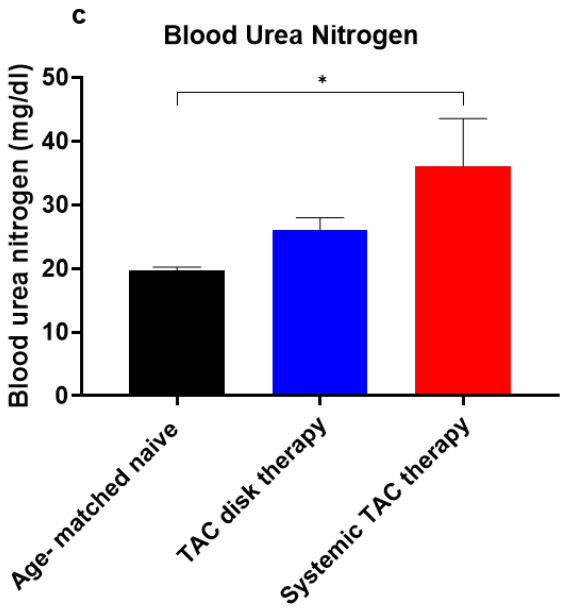


**Figure 50. TAC disk therapy did not induce hepatic or renal toxicity. Biochemical analyses of the levels of (a) aspartate aminotransferase AST, (b) alanine aminotransferase ALT, and (c) blood urea nitrogen BUN in blood collected at post-operative day 120 from naïve (age matched) animals or transplanted animals received TAC disk therapy. Data are shown as mean ± SD. P values were calculated by one-way ANOVA with Tukey's multiple comparisons test. Significant differences are indicated by *p < 0.05, *** p < 0.001, and **** p < 0.0001.**

### 5.4.9 Single TAC-Eluting Disk in the Allograft does not Induce Hyperglycemia or Diabetes

To test whether TAC disk therapy could result in hyperglycemia or diabetes, the blood glucose levels was evaluated. As shown in **Figure 51 (a)**, the average glucose levels in the transplanted animals received no treatment or received TAC disk therapy (Group 4) were in the normal range (80-100 mg/dl) at all time points. Transplanted animals received intraperitoneal injection of TAC in a dose of 1mg/kg/day (Group 2) showed a trend toward increased glucose levels in the first month post-transplantation, and the levels remained high and achieved the highest values (112-120 mg/dl) during the period 60-120 days post-transplantation. TAC disk vs. Un-treated group ($p>0.1$) at all time points. Glucose levels of the intraperitoneal glucose tolerance test (IPGTT) are shown in **Figure 51 (b).** All transplanted animals in systemic TAC group developed prediabetes as indicated by glycemia at 120 min (IPGTT) >140 and <200 mg/dl. All transplanted animals in TAC disk group didn't develop diabetes or prediabetes as indicated by glycemia at 120 min (IPGTT) <140 mg/dl (121±14.9 mg/dl). TAC disk vs. systemic TAC group (****$p<0.0001$) at all time points post IPGTT. Total systemic glucose exposure in the animals received systemic TAC therapy were markedly higher than the values observed in the animals received no treatment or TAC disk therapy (22631± 496.7mg.min/dL vs. 14479±465.4 and 16399 ± 706.3 mg.min/dL, respectively. *$p<0.05$).

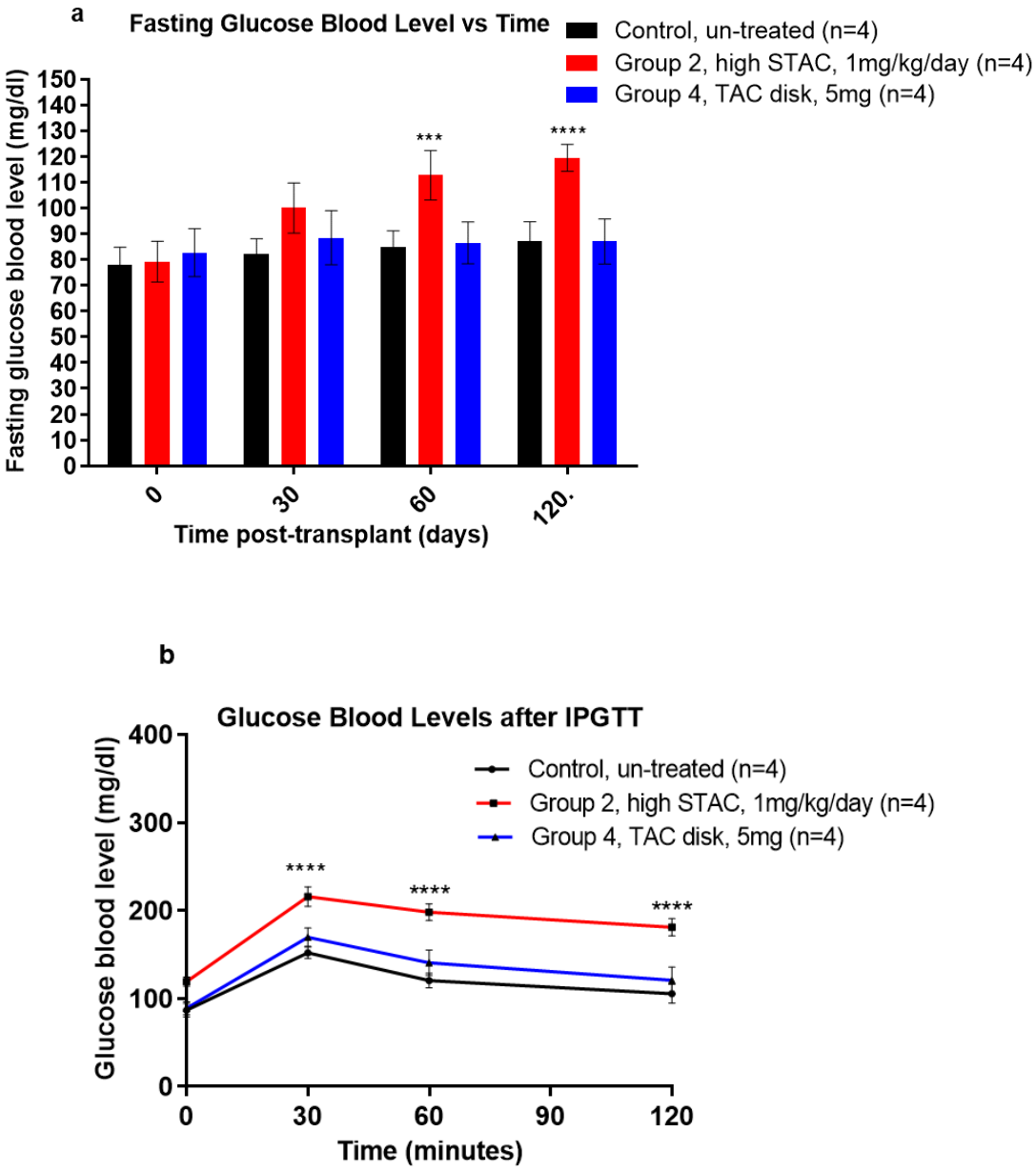


**Figure 51. TAC disk therapy did not result in hyperglycemia. (a). Average glucose levels (mg/dl) in transplanted animals received no treatment (n=4) or a single TAC disk (n=4) or intraperitoneal injection of TAC in a dose of 1mg/kg/day (n=4) at specific time points during the study period. (b). Average glucose levels (mg/dl) after IPGTT performed on post-operative day 125. Statistical analyses between the groups are shown. Data shown as mean+SD. P values were calculated by repeated measure ANOVA. Significant differences are indicated by, *** $p < 0.001$ or **** $p < 0.0001$.**

## 5.5 Discussion

Site-specific delivery of TAC to the allograft and DLNs can provide high loco-regional drug concentrations to inhibit the alloimmune response, prevent rejection, and prolong the survival of VCA allografts, while limiting the complications of systemic nonspecific immunosuppression. Previous work has shown that single TAC-loaded double-walled microsphere (PLGA-PLA) disk implanted in the transplanted hind limb maintained allograft survival (100%) for 180 days [106]. In this study, a recipient with an average weight of 300 g would have received 40 mg of TAC. There was initial burst release of TAC leading to high levels (21.2 ± 5.8 ng/ml) followed by 5-15 ng/ml throughout the study. PLGA and PLA undergo hydrolytic degradation and produce lactic and glycolic acid and induce inflammatory response and fibrotic capsule formation [255, 256]. These necessitated re-designing of the disk to better control the drug release to lower the overall systemic drug exposure, and to evaluate whether rejection can be prevented or treated by local TAC alone. In this study, we developed a novel technology of drug-eluting biomaterials that can be placed within allograft. This technology can control drug release over prolonged period of time. This results in lower overall systemic drug exposure, while the sustained loco-regional delivery facilitates VCA survival. Our goal was to promote VCA allograft survival and to minimizes the risk of systemic side effects of immunosuppressive drugs.

Lew and BN rats are a complete MHC mismatch resembling the clinical situation. Animals received one TAC disk either in the transplanted (DTx) with or without lymphadenectomy, or in the contralateral non-transplanted (DnonTx) limbs. Allografts survival was evaluated and compared to assess the efficacy of sustained site-specific immunosuppression using a single TAC-eluting disk within allografts. Concentrations of TAC in the blood and the allograft were measured to determine the effective concentrations that need to be achieved in these tissues to prevent the

rejection. Glucose levels and creatinine clearance were measured to assess the systemic toxicity. The immunomodulatory effects of TAC were studied to identify the mechanisms that are responsible for maintaining the allograft survival.

Polycaprolactone as is a biodegradable polymer, that is biocompatible with tissues and most of drugs, that is approved by FDA for clinical use and doesn't produce local acidic environment, was determined to be a suitable biomaterial to deliver TAC. TAC was added to the polymeric mixture of PCL to form a 3-dimensional porous structure with a spherical disk shape, at was hard enough to hold the physical compression which allows it to be easily handled and cut into different shapes and sizes, to facilitate the loco-regional drug delivery. Only five milligrams of TAC were loaded to the disk to reduce the initial burst release and the overall systemic drug exposure, while maintaining loco-regional drug concentrations for a prolonged period. PCL and PCL-TAC disks exhibited a slow degradation rate with low mass loss which indicates that the disk can stay intact for few months. This is related to the semi-crystalline characteristics of PCL that prevents its degradation in solution [256, 257]. In-vitro, TAC was released in a gradual and sustained manner for one month with reduced initial burst in the first 24-48hr. The gradual and sustained release of TAC from PCL disk is attributed to the entrapment of TAC into PCL, which controls the release of TAC. In-vitro, the cumulative amount released was linearly with time, which is desirable to achieve a prolonged pharmacological activity. Only thirty seven percent of TAC loaded in the disk was released in one month, suggesting that the amount loaded could be sufficient for approximately 3 months. This technology enables prolonged retention and bioavailability of TAC, thus allowing the drug to be released at low rates. This helps to exerts loco-regional immunosuppressive effect by TAC to promote hind limb survival.

Our results show that sustained site-specific immunosuppression using a single TAC eluting disk within allograft was effective in inhibiting rejection and sustaining VCA allograft survival (>150 days). On the other hand, contralaterally implanted TAC disks were less effective, suggesting that the immunosuppressive effect is exerted locally by TAC at the transplant site. Additionally, low blood levels of TAC were observed following implantation of TAC disk at the transplanted limbs, indicating that the high levels of TAC in the blood are not necessary for the allograft survival.

Tacrolimus concentrations were significantly higher in the disk implanted limbs (allografts), compared to the concentrations observed in the blood and in the contralateral non-transplanted limbs. These findings indicate that the allograft survival could be related to the long-lasting high loco-regional concentrations of TAC at allograft and DLNs (target sites for rejection), and these concentrations were effective in inhibiting the local alloimmune response and maintaining the allograft survival. Histological observation confirms the absence of any signs of rejection in the skin and muscle.

The elevated blood levels of TAC that were observed initially in the first week inhibited the overwhelming systemic alloimmune response early after transplantation, and the local TAC inhibited the ongoing local alloimmune responses. The average weekly concentrations of TAC in the blood was significantly lower than the values observed in the animals that received systemic TAC therapy (3±2 vs 10±2ng/ml, $p<0.05$).

The lipophilicity of TAC (log p = 3.9), disk location, local drug diffusion to the surrounding tissues, and the prolonged systemic TAC exposure contributes to the availability and deposition of TAC in the local tissues [258]. Single TAC-eluting disk implanted in the transplanted limbs (group 4) results in significantly higher locoregional concentrations of TAC in the allograft tissues

such as DLN, muscle, and skin, when compared to the concentrations observed in the blood (260, 123, and 34-fold higher), and when compared to the contralateral tissues (4, 6, and 3-fold higher). However, DLNs appeared to retain most of the drug when levels across skin, muscle, and DLNs were compared. This is consistent with our previous findings and indicates the high affinity of TAC for the deposition into the fat tissues [258-260]. Additionally, the concentrations that are observed in the surviving allografts are effective therapeutic concentrations that should be maintained to prevent the rejection along with minimal systemic levels of TAC.

Single TAC-eluting disk implanted in the contralateral non-transplanted limb (group 3) results in significantly higher locoregional concentrations of TAC at the contralateral non-transplanted limb particularly the DLNs, when compared to the concentrations observed in the blood and in the allograft DLNs, muscle, and skin. Contralateral non-transplanted limb DLNs, muscle, and skin had significantly higher TAC concentrations when compared to the blood (147, 87, and 29-fold higher) and to the allograft DLNs, muscle, and skin (7, 4.4, and 4-fold lower). The low concentrations of TAC in the allograft tissues (19±5ng/g for skin, 58±23ng/g for muscle, 70±34ng/g for DLNs) along with the low blood levels (2.5±1.5ng/ml) were insufficient to maintain the allograft survival over a prolonged time. This indicates that the effective concentrations that should be achieved in the allograft tissues to prevent the rejection should be higher than these concentrations. Tacrolimus was also detected in the local sciatic nerve. Studies has been shown that TAC could hasten recovery and improve functional outcomes in animal models as well as in hand transplant patients [261].

Our results demonstrate that PBMCs of animals that received TAC disk in the transplanted or non-transplanted limbs showed normal or slightly activated lymphocyte proliferative response to donor PBMCs as compared to control naïve animals. However, the animals that received TAC

disks in the contralateral non-transplanted limbs rejected their allografts by day 70 while animals that received disks in the transplanted limbs maintained their allografts (>150 days). This indicates that locally delivered TAC (with a single TAC disk) was capable of sustaining the allograft survival by immunosuppression at the local transplant site and without affecting the systemic alloimmunity. Furthermore, the levels of hematopoietic chimerism and T regulatory cells in peripheral blood appeared unaffected with TAC-eluting disk within the allograft. However, TAC acts by inhibiting T eff cells mediated immune response and does not induce T regs expansion and tolerance. This indicates that the allograft survival was not related to the induction of donor-specific tolerance but to the long-lasting high loco-regional concentrations of TAC in the allograft.

Our results demonstrate that animals that received TAC disks in the transplanted limbs, removal of DLNs in the groin of the transplanted limb delayed the rejection. However, all animals eventually rejected their allografts by day 175. This may be due to dendritic cell/T-cell interaction that occurs at other remote secondary lymphoid tissue, that subsequently induces rejection in the absence of TAC locally at those sites. This indicates that DLNs are important sites and targeting the allograft and DLNs with immunosuppressive drugs can improve the allograft survival.

Another goal of our current drug delivery system is to reduce the toxicity of immunosuppressive drugs. The nephrotoxicity of TAC has been well studied and is one of the most serious side effect in transplant patients [229]. It is associated with the systemic blood levels of TAC [262]. Therefore, maintaining the concentrations within the targeted therapeutic range (5-10ng/ml) can reduce the risk of nephrotoxicity [79, 230, 263]. Clinically, the nephrotoxic effect of calcineurin inhibitors (TAC and cyclosporine) is a significant challenge in kidney and liver transplantation and represents a barrier to successful long-term clinical outcomes [230]. VCA transplant recipients are at a high risk of systemic side effects including nephrotoxicity, because

they undergo long-term immunosuppression at higher doses to prevent the rejection of highly antigenic tissues [215, 264]. Experimentally, it has been reported that short-term exposure (2 weeks) to TAC orally in a dose of 3 mg/kg/day significantly decreased the glomerular filtration rate in Sprague–Dawley rats as compared to controls [225]. Short-term exposure (1 week) to TAC subcutaneously in a dose of 0.2, 0.4, and 0.8 mg/kg/day had slightly reduced GFR to 83, 74, 72% respectively of the controls value, while long term exposure (4 weeks) to the same doses, had significantly reduced GFR to <30% of the control value ($P<0.001$) [231].

Our results show that the transplanted animals that received TAC disk did not show significant change in the creatinine clearance and blood urea nitrogen levels, and those were similar to the values obtained in the animals received no treatment. Transplanted animals that received daily systemic TAC therapy (total TAC amount given intraperitoneally for 150 days was 45 mg for a 300g rat), showed signs of nephrotoxicity as indicated by the significantly decreased creatinine clearance to 50% of the values observed in the animals received no treatment. ($p<0.05$), and by the significantly elevated levels of BUN as compared to the naïve (age matched) animals ($p<0.05$). Similarly, the transplanted animals received daily systemic TAC therapy showed signs of hepatotoxicity as indicated by elevated levels of AST. These results are consistent with other studies [225, 265, 266], and confirmed our hypothesis that locally administered tacrolimus within allografts sustains allograft survival with a lower risk of systemic toxicity [249].

Our results show that transplanted animals that received TAC disk did not develop hyperglycemia, where the treatment resulted in estimated trough mean levels of TAC between 2 and 5 ng/ml until postoperative day 105. After this, the concentrations dropped to less than 2 ng/ml. Our results are consistent with our previous study where topical therapy in conjunction with low dose of systemic TAC did not develop hyperglycemia, where the treatment resulted in estimated

trough mean levels of TAC 2.8±1ng/ml. While high systemic TAC therapy (1mg/kg/day) induced hyperglycemia during the period between 60-120 days post-transplantation, where the treatment resulted in estimated trough mean levels of TAC of 10±2 ng/ml. This confirms that the diabetogenic effect of TAC is dose and time dependent [231]. Studies showed that TAC has inhibitory effect on insulin secretion [237].This explains the elevated glucose levels and the impaired glucose tolerance, that was observed in the animals received high systemic TAC. However, there was no significant change in the blood glucose levels in the animals received TAC disks as compared to the initial levels.

Additionally, TAC disks were implanted subcutaneously in the hind limbs of rats, which allows for the local delivery of TAC into the transplanted limbs, and for the ease of removal of the disk in case of any observed complications including infections or malignancies. Our system sustained allograft survival by loco-regional immunosuppression, reduced overall systemic drug exposure and associated systemic side effects, minimizing the need for daily intake of (oral and topical) immunosuppressive agents.

# 6.0 Summary and Future Directions

## 6.1 Summary of Major Findings and Conclusions

The high incidence of rejection and the requirement for systemic, lifelong, high-dose, multi-drug maintenance immunosuppression, are the main factors preventing a wider clinical application of VCA. Site-specific immunosuppression using safe and effective local drug delivery strategies and formulations is a potential alternative approach that may help to overcome these challenges, increasing the therapeutic efficacy and patient compliance while reducing systemic toxicity. Herein, we have described two strategies that can provide site-specific immunosuppression that were able to sustain VCA survival with minimal systemic immunosuppression and without systemic side effects in a rat model of VCA.

First chapter included a background and introduction to the dissertation work. In Chapter 2.0 (Topical tacrolimus: evaluation of systemic exposure and tissue concentrations), we evaluated the ability of topical TAC (Protopic® 0.03%) at a dose of 0.5mg/kg to provide high tissue concentrations at the site of application for local immunosuppressive effect while minimizing systemic exposure and consequentially any off-target effects. We evaluated the blood levels and tissue concentrations of topical tacrolimus (Protopic® 0.03%, 0.05mg/kg) following single and repeated topical application in comparison to those after systemic administration in rats. Results of this study show that topical application of TAC ointment (Protopic®, 0.03%) once daily at a dose of 0.5 mg/kg provides high concentrations in the skin and confirms the feasibility of targeting specific tissues by topical delivery of drug with minimal systemic exposure. Systemic exposure to TAC following topical application was low. Apparent topical bioavailability of TAC was 11% of those achieved after systemic administration. Local tissue concentrations (skin, muscle, and DLNs) were significantly higher than the values observed after systemic administration of the same dose (300, 66, and 43-fold higher, respectively). The highest concentrations of TAC were

observed in the skin, which is desirable as the skin is the primary target tissue for rejection in VCA. The local accumulation of TAC after repeated applications can prolong drug effect because tissues release the drug slowly as blood levels decline. TAC ointment (Protopic®, 0.03%) is a well-tolerated formulation for local delivery of TAC. There were no topical therapy related side effects. This is consistent with the results of other studies where topical TAC 0.03% mainly partitioned in the skin, with minimal systemic absorption in patients with atopic dermatitis. Further studies should be performed to evaluate the efficacy of TAC (Protopic® 0.03%) in preventing skin rejection in a rat model of VCA.

In Chapter 3.0 (Topical MPA: preparation of formulation, and evaluation of pharmacokinetics and bioavailability), we report the preparation of a topical formulation of MPA in Lipoderm (1%w/w). We evaluated in-vitro/in-vivo characteristics of the formulation such as release, permeation, and blood and tissue bioavailability to enable safety and efficacy evaluation in clinical VCA. In-vitro permeation studies were performed with different semisolid formulations of MPA (1%w/w) including Aladerm, Lipoderm, Emollient, and Versa Base using a franz diffusion cell system. In-vivo studies were performed to evaluate the systemic exposure and tissue concentrations of MPA in Lipoderm (1%w/w) following single or repeated topical application in comparison to those after systemic administration in rats. We evaluated the ability of topical MPA (Lipoderm 1%) at a dose of 16.6mg/kg/day to achieve high tissue concentrations at the site of application for local effects without or with low systemic concentrations. Results of this study show that MPA (Lipoderm 1%) exhibits optimal invitro release profile as the formulation of choice for topical application in clinical VCA including gradual diffusion and sustained drug release which facilitates a prolonged local site-specific action of the drug. The prepared formulation exhibits good physical characteristics and stability over 6 months of storage at 25 °C, and provides

a safe topical delivery system. In-vivo, topical application of MPA (Lipoderm 1%) once daily at a dose of 16.6mg/kg/day provides high local concentrations in the skin and confirms the feasibility of targeting specific tissues by topical delivery of drug with minimal systemic exposure. Topical bioavailability of MPA was 9% of those achieved after systemic administration. The highest concentrations of MPA were observed in the skin, which is desirable because skin is the primary target tissue for rejection in VCA. However, the dosing regimen can be further modified to minimize drug accumulation after repeated topical applications. MPA in Lipoderm (1%w/w) is well-tolerated for local delivery of MPA.

In Chapter 4.0 (Safety and efficacy study of combined topical immunotherapy in conjunction with low systemic immunotherapy), we evaluated whether combined treatment of topical TAC and MPA applied on the transplanted site in conjunction with low dose systemic immunosuppression with TAC can be effective in sustaining the survival of the VCA graft and in reducing systemic morbidity in a rat model of VCA. Allograft survival was evaluated clinically and histologically. Blood and allograft tissue concentrations of TAC were measured using LC-MS/MS. Systemic toxicity was evaluated by measuring blood glucose levels and creatinine clearance. The immunomodulatory effect of TAC and MPA was assessed by flow cytometry and secondary skin grafting. Results of this study show that once daily combined treatment of topical (TAC+MPA) therapy applied at the allograft prevents skin rejection and prolongs the survival of VCA grafts (>100 days) with low systemic immunosuppression and without systemic side effects. This finding has been confirmed histologically by the absence of any sign of rejection in the skin and muscle. Whereas applying topical (TAC+MPA) therapy on the contralateral non-transplanted limbs was less effective, suggesting that the immunosuppressive effect is exerted locally by TAC and MPA at the transplant site with minimal influence by the systemic drug levels. Furthermore,

low “sub-therapeutic” blood levels of TAC were observed during the study duration indicating that the high levels of TAC in the blood are not necessary for the allograft survival. Long-lasting high locoregional concentrations of TAC and MPA was observed in the allograft skin, muscle, DLNs. Our results are consistent with other studies and confirmed our hypothesis that topical administration of TAC and MPA directly to the allografts provides sustained high locoregional drug concentrations to effectively inhibit the local immune response (donor allograft), and this enables a reduction of systemic immunosuppression levels.

The low concentrations of TAC and MPA in the allograft tissues (TAC: 22±18ng/g for skin, 36±13ng/g for muscle, 28±11ng/g for DLNs; MPA: 2±1µg/g for skin, 2.4±1.1µg/g for muscle, 7.5±1.1µg/g for DLNs) along with the low blood levels of TAC (2.6±1ng/ml) were insufficient to maintain the allograft survival over a prolonged time. This indicates that the effective concentrations that should be achieved in the allograft tissues to prevent the rejection should be higher than these concentrations. Topical application of TAC (Protopic®, 0.03%) at a dose of 0.5mg/kg and MPA (Lipoderm, 1%) at a dose of 16.6mg/kg once every 2 days can be effective in reducing the local drug accumulation associated with daily application and in achieving and maintaining the effective therapeutic tissue concentrations for local effects with low systemic exposures. The systemic exposure (troughs) and local tissue concentrations of TAC and MPA at the application site were higher when the topical formulations were applied on the transplanted limbs, as compared to the concentrations observed when the topical formulations were applied on the non-transplanted limbs with intact healthy skin. However, the difference was not significant. This indicates that transplantation (surgical inflammation) has a minimal effect on the systemic drug absorption and exposure.

Combining TAC and MPA exhibited inhibitory effect on T cell response as indicated by the significantly decreased lymphocyte infiltration in the skin, while preserving the circulatory Treg levels. The allograft survival was not related to the induction of donor-specific tolerance but to the long-lasting high loco-regional concentrations of TAC and MPA in the allograft. The combined treatment did not show sign of nephrotoxicity or metabolic complications as indicated by the normal creatinine clearance and glucose blood levels. Combined treatment of topical therapy (TAC and MPA) applied on the VCA graft with low dose of STAC can be an effective therapeutic strategy to sustain VCA graft survival and reduce systemic side effects. Our study provides rationale to shift the current immunomodulatory paradigms of systemic immunosuppression to loco-regional immunosuppression using a combination immunosuppression drug therapy approach.

In Chapter 5.0 (Safety and efficacy study of Tacrolimus disk), we developed a drug-eluting disk that can be placed in the allograft that can release TAC in a controlled manner over prolonged period and evaluated the efficacy of such delivery system in preventing rejection and sustaining VCA allograft survival without systemic side effects. The results of this study show that controlled delivery of TAC directly to the allograft and DLNs (with a single TAC disk) effectively inhibits rejection and prolongs VCA allograft (>150 days) without signs of metabolic, infectious, or neoplastic complications. In these animals, TAC levels in blood were low but stable between 2 to 5 ng/ml for nearly 100 days. Long-lasting high loco-regional concentrations of TAC were achieved in the allograft tissues particularly DLNs. DLNs, muscle, and skin had significantly higher TAC concentrations when compared to the blood (220, 123, and 34-fold higher) and when compared to the contralateral tissues (6, 6, 3-fold higher). However, DLNs appeared to retain most of the drug when levels across skin, muscle, nerve, and DLNs were compared. This is consistent with our

previous findings [258-260]. These findings indicate that the allograft survival could be related to the long-lasting high loco-regional concentrations of TAC at allograft and DLNs (target site for rejection), and these concentrations were effective in inhibiting the local alloimmune response and maintaining the allograft survival with minimal systemic levels.

TAC disk in the contralateral non-transplanted limbs results in significantly higher locoregional concentrations of TAC at the non-transplanted limb particularly the DLNs, when compared to the concentrations observed in the blood. The low concentrations of TAC in the allograft tissues (19±5ng/g for skin, 58±23ng/g for muscle, and 70±34ng/g for DLNs) along with the low blood levels (2.5±1.5ng/ml) were insufficient to maintain the allograft survival over a prolonged time. This indicates that the effective concentrations that should be achieved in the allograft tissues to prevent the rejection should be higher than these concentrations.

Systemic lymphocyte proliferative response appeared unaffected with local TAC. This indicates that locally delivered TAC (with a single TAC disk) was capable of sustaining the allograft survival by immunosuppression at the local transplant site and without affecting the systemic alloimmunity. Furthermore, the levels of hematopoietic chimerism and T regulatory cells in peripheral blood appeared unaffected with TAC-eluting disk in the allograft. This indicates that the allograft survival was not related to the induction of donor-specific tolerance.

Controlled delivery of TAC directly to the allograft (with a single TAC disk) over a prolonged period effectively inhibits rejection and significantly prolongs VCA graft survival, while mitigating the complications of systemic immunosuppression. There was a profound survival benefits of delivering TAC within the allograft as compared to a remote site. Loco-regional immunosuppression offers an alternative to the current treatment paradigms which generally utilizes systemic immunosuppression. Localized delivery of TAC to the allograft and

DLNs may create immunosuppressive environment to maintain VCA survival. Our findings suggest that localized delivery of TAC to the allograft could be a safe and effective strategy for promoting VCA allograft survival and minimize daily systemic immunosuppression. Loco-regional immunosuppression is worth more investigation, through basic research to establish a mechanism to regulate graft immunity and through clinical research to achieve optimal immunosuppression with reduced toxicity. This will not only improve patient compliance and quality of life but will also increase the clinical applicability of VCA.

## 6.2 Limitations

While promising data were generated through this dissertation research, there were limitations to the performed dissertation work that should be recognized and considered.

In Vitro studies: Franz cells diffusion system with artificial skin was used to assess the in-vitro release and permeation of drugs across membranes after topical application. However, artificial skin is not fully representative of human skin. Ex-vivo human skin or pig skin are better surrogates for in vivo human skin permeation and can provide results that have better correlation with in-vivo results.

Species difference in drug skin permeability: Rats are commonly used in preclinical studies such as safety and pharmacokinetics. However, the differences in the skin structure, thickness, and permeability between rats and human should be considered. The actual systemic exposure in human may be over or under-estimated if the assessment was made based only on the results of the animal studies.

Tissue concentrations: Tissue concentrations are determined by homogenizing the whole tissues. This does not take into account that tissues are composed of different components (extracellular fluid, intracellular fluids, and cells) and skin is composed of several layers (epidermis, dermis, and hypodermis) in which the drug may not be unformly distributed.

Intravenous administration was used to evaluate the systemic bioavailability of the drug after single dose topical application. Intraperitoneal administration was used to evaluate the systemic exposure (troughs) and local tissue concentrations after repeated systemic administration as compared to the systemic exposure (troughs) and local tissue concentrations after repeated topical applications. Intraperitoneal route is safer and more convenient to conduct studies that require daily dosing for a period of time. Additionally, we used the same route of administration

that we use in the actual efficacy studies (Intraperitoneal administration for multiple once daily doses).

Inter-animal differences: To evaluate the time course of TAC concentrations in skin and muscle over 24 hours after topical application, several samples should be taken from the same application site at multiple time-points from the same animals which is impractical. In our study, the samples were taken at multiple time-points from different animals. However, this may increase the likelihood of variability due to the inter-animal differences.

Single treatment: TAC and MPA formulations were applied at different times in order to minimize the risk of transdermal drug interaction (e.g. altered release kinetics) and systemic absorption due to concurrent application of TAC and MPA formulations, Tacrolimus ointment was applied 12 hours after MPA in Lipoderm. However, combined topical product (TAC+MPA) should be considered in future work.

Individual drug vs. combination drug study: Studies in Chapter 2 and 3 were performed with single topical agent. However, combination drug study should be performed to evaluate the transdermal drug interaction (e.g. alter release kinetics) and systemic absorption due to concurrent application of TAC and MPA formulations.

Currently, TAC is commercially available in only two concentrations (Protopic® 0.03% and 0.1%). We typically use a fixed amount (0.5g) of ointment to completely cover the whole surface area of the rat hind limb (26 $cm^2$). To reduce the dose, we need to either develop a new formulation with a lower concentration or dilute the current formulation with the same ointment base. In both cases, we need to evaluate the release, permeation, and pharmacokinetics of the new formulation.

Altered drug permeability: Pharmacokinetic studies were performed in naïve rats with intact healthy skin in order to evaluate the dermal absorption of TAC from the topical formulation. However, systemic absorption and exposure is expected to be different in compromised skin as compared to intact healthy skin. Transplantation or surgical inflammation may increase absorption and exposure. Therefore, pharmacokinetic studies should be performed in the transplant model to evaluate the effect of transplantation or surgical inflammation on the drug absorption and exposure after repeated topical application.

Minimum dose required: The minimum therapeutic concentrations of TAC in the VCA graft particularly skin that are required to prevent the rejection is unknown. In our studies, we developed formulations for local drug delivery to the VCA graft and we assessed the relationships between systemic drug levels, tissue drug levels, and the outcomes. Topical application of TAC (Protopic®, 0.03%) at a dose of 0.5mg/kg and MPA (Lipoderm, 1%) at a dose of 16.6mg/kg once every 2 days can be effective in maintaining the effective therapeutic tissue concentrations for local effects with low systemic exposures. Controlled delivery of TAC directly to the allograft (with a single disk loaded with 5 mg of TAC) can be effective in maintaining the effective therapeutic tissue concentrations for local effects with low systemic exposures in small animal model of VCA. However, more studies are needed to determine and/or confirm minimal therapeutic concentrations of TAC that should be achieved in the blood and VCA graft to maintain the allograft survival.

Chronic topical treatment of rats with TAC causes systemic side effects including changes in the body weight and kidney function. These changes are due to the increased systemic absorption and exposure after repeated topical application. In our pharmacokinetic study, we evaluated the effect of TAC (0.03% ointment, 0.5mg/kg) only on the body weight change after

short-term treatment with TAC for 7 days. Assessment of the body weight and kidney function should be performed after long-term treatment with topical TAC to confirm the safety and tolerability of the formulation.

Daily topical application of immunosuppressive drugs: The major challenge in topical application of immunosuppressive drugs is ensuring that the drug can be applied to the entire transplanted limb in order to treat rejection while preventing the drug from entering other areas of the body through blood stream, which could lead to systemic exposure and systemic adverse effects.

Topical agent: The practical applications of topical agents as part of the standard routine care in clinical VCA, is associated with some challenges including the need for multiple daily applications that require high patient compliance, limited skin penetration for drugs with large molecular weight such as cyclosporine, and lack of commercially available topical formulations for immunosuppressive drugs such as rapamycin.

Sample size: We had four to five animals in some of the groups tested. Some animals were lost or excluded due to technical failure or surgical complications post-operation. In the future a minimum 6 animals for each group should be evaluated.

TAC disk can be subcutaneously implanted during surgery and while patient is under anesthesia. However, surgical intervention is required to remove these drug delivery systems when systemic complications are observed or to re-implant a new drug delivery system after drug exhaustion.

Control groups: Treated animals with vehicle (no drug) or with empty disks are needed to evaluate the effect or contribution of the vehicle and/or the empty disk on the animal and the allograft survival.

## 6.3 Future Research Directions

Topical MPA (Lipoderm 1%): Future in-vitro studies are being planned to fully evaluate the long-term stability of this formulation in order to determine the shelf life and optimal storage conditions. Formulation with lower concentrations (0.5% and 0.3%) should be prepared to further minimize the risk of systemic exposure with repeated topical application.

Combined topical MPA and topical TAC: Future studies should evaluate the transdermal drug interaction (e.g. release kinetics) and systemic absorption of a combination of TAC and MPA in one formulation.

Other drug combinations: MPA in lipoderm can be combined with other topical immunosuppressive drugs such as rapamycin for additive or synergistic efficacy on T cell responses. Optimization of topical MPA formulations could thus lead to effective combination topical immunosuppression protocols (+ RAPA +/- TAC) for site-specific therapies (+/- low dose systemic immunosuppression) in VCA to prevent AR or CR. In addition, combination therapy can result in more rapid onset, increased efficacy, reduction of side effects, and thus improving the outcomes.

Mechanistic: In addition to local and systemic exposure measurements, the immunomodulatory effect of our treatment strategy on the cytokines and immune cells (donor allograft and recipient blood) implicated in the rejection over the period of treatment should be studied.

Drug distribution in VCA: Skin particularly epidermis is the most immunologically susceptible tissue in the VCA grafts and the target for rejection. Concentrations of drug in each skin layer can be measured by using the tape-stripping technique.

Large animal VCA studies: Our results were obtained in preclinical experiments in small animal model of VCA and showed that site-specific immunosuppression is a feasible and effective alternative approach to control allograft rejection and reduce systemic toxicity. The next step is to evaluate the efficacy of the drug delivery systems in sustaining the allograft survival with site specific immunosuppression in a large animal model of VCA. Further studies should be performed to further optimize the dose of TAC in disk and/or the dosing regimen of topical immunosuppressive drugs to maintain the effective therapeutic concentrations in the VCA graft tissues with low systemic immunosuppression. The results of these studies will help to develop novel drug delivery strategies and formulations for therapeutic delivery in VCA, and to design future clinical trials in VCA patients. Moreover, future work is required to develop and optimize a system that can provide minimally invasive site-specific drug delivery to solid organ grafts.

Clinical VCA studies: It is also important to evaluate the potential of translating the results obtained in preclinical experiments (small and large animal model of VCA) into clinical VCA. Topical TAC is FDA-approved and commercially available (Protopic™) in two concentrations 0.1%, 0.03% (Astellas Pharma Inc) for certain dermatological conditions, and has been used successfully in VCA, off-label, to treat AR episodes. MPA in Lipoderm is a well-tolerated formulation for local delivery of MPA. Polycaprolactone as is a biodegradable polymer, that is biocompatible with tissues, that is approved by FDA for clinical use, was determined to be a suitable biomaterial to deliver TAC. TAC was added to the polymeric mixture of PCL to form a 3-dimensional porous structure with a spherical disk shape, at was hard enough to hold the physical compression which allows it to be easily handled and cut into different shapes and sizes, to facilitate the loco-regional drug delivery to the transplanted organ. PCL and PCL-TAC disks exhibited a slow degradation rate which indicates that the disk can stay intact for few months. Our

results confirm the feasibility of these drug delivery systems for site-specific immunosuppression and enable further safety and efficacy evaluation in clinical VCA. Further studies should be performed to further optimize the dose of TAC in disk and/or the dosing regimen of topical immunosuppressive drugs to maintain the effective therapeutic concentrations in the VCA graft tissues with low systemic immunosuppression. With this research, we hope to establish the basis for the development of more advanced systems or alternative formulations of site-specific immunosuppression that could help maintain allograft survival, minimize overall exposure (dosing, timing, and frequency) of systemic immunosuppressive drugs and improve patient compliance and quality of life.

On demand drug release: Considering the immunologic features of VCA, there is a need not only for local drug delivery to avoid systemic drug toxicity, but also for the ability to modify or use different drug doses over short periods to control or inhibit the immune response and prevent the rejection. A drug delivery system that can not only provide sustained local drug release but also on demand drug release in response to specific stimuli such as enzymes that are significantly upregulated during inflammation or rejection may facilitate long-term VCA survival.

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